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<b>(21) International Application Number:</b> PCT/US91/01726 <b>(22) International Filing Date:</b> 14 March 1991 (14.03.91)  <b>(30) Priority data:</b> 494,093 15 March 1990 (15.03.90) US  <b>(60) Parent Application or Grant</b> (63) Related by Continuation US 494,093 (CIP) Filed on 15 March 1990 (15.03.90)  <b>(71) Applicant (for all designated States except US):</b> THE BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West Seventh Street, Austin, TX 78701 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> SAXENA, Inder, M., Jr. [IN/US]; 4559 Avenue A #201, Austin, TX 78751 (US). LIN, Fong, Chyr [-/US]; 3555-A Lake Austin Blvd., Austin, TX 78703 (US). BROWN, R., Malcolm, Jr. [US/US]; 305 Skyline Drive, Austin, TX 78746 (US).		<b>(74) Agent:</b> PARKER, David, L.; Arnold, White & Durkee, 750 Bering Drive, P.O. Box 4433, Houston, TX 77210 (US).  <b>(81) Designated States:</b> AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> RECOMBINANT $\beta$ -1,4 GLUCAN SYNTHASE PROTEINS		
<b>(57) Abstract</b> <p>The present invention relates to the compositions and methods associated with the cloning of the subunits of <math>\beta</math>-1,4 glucan synthases responsible for catalyzing <math>\beta</math>-1,4 glucan polymer biosynthesis. The invention relates further to compositions and methods for obtaining host cells containing recombinant <math>\beta</math>-1,4 glucan synthases as well as compositions and methods for obtaining <math>\beta</math>-1,4 glucan synthase from natural sources. In certain aspects, the present invention provides methods for the cloning of both the 83 Kd subunit and the 93 Kd subunit of the cellulose synthase enzyme from <i>Acetobacter xylinum</i>.</p>		

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**RECOMBINANT  $\beta$ -1,4 GLUCAN SYNTHASE PROTEINS**

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This invention relates generally to compositions of and methods for obtaining  $\beta$ -1,4 glucan synthase. The  
20 invention relates as well to the DNA sequences encoding  $\beta$ -1,4 glucan synthase, the recombinant vectors carrying those sequences, the recombinant host cells including either the sequences or vectors and recombinant  $\beta$ -1,4 glucan synthase polypeptides. More specifically, the  
25 invention relates to the cloning and expression of  $\beta$ -1,4 glucan synthase from Acetobacter xylinum and cotton.

$\beta$ -1,4 glucan biosynthesis such as cellulose biosynthesis is an event largely associated with plant  
30 cells, especially certain agronomic species such as cotton where as much as 90-95% of the secondary wall of mature cotton fibers is composed of cellulose. However, studies of  $\beta$ -1,4 glucan biosynthesis in higher plants have been very frustrating, mainly because  $\beta$ -1,4 glucan synthase,  
35 due to its lability, has evaded isolation and purification. Furthermore, the study of this enzyme has been hampered by the great difficulty in detecting formation of  $\beta$ -1,4 glucans such as cellulose in vitro using purified preparations of plant and other eukaryotic

-2-

cells [Delmer 1987: In higher plant preparations, the  $\beta$ -1,3-glucan (callose) chains are synthesized instead of the  $\beta$ -1,4-glucan of cellulose]. Frost et al. (1990) have identified a 57 Kd band which was partially purified by product entrapment and is believed to represent the

Apart from the higher plants, a large number of bacteria synthesize  $\beta$ -1,4 glucans such as cellulose, including Acetobacter xylinum (A. xylinum), which converts as much as 35% of the glucose supplied to cultures of this bacterium into cellulose. Unlike the situation in plants and other eukaryotic cells noted above, the cellulose synthase of this bacterium has been isolated to a certain degree. Because of these facts, coupled with the capacity of the bacterium to synthesize a highly purified form of cellulose in vitro using membrane preparations, A. xylinum has become a preferred system for studies on the synthesis and organization of cellulose [Delmer 1987: In the bacterium,  $\beta$ -1,4-glucan (cellulose) chains can be detected].

The biosynthesis of cellulose in A. xylinum is visualized as a two-step process. The first step involves the polymerization of sugar nucleotides (UDP-glucose; UDP-glc) into a  $\beta$ -1,4,-linked glucan chain. The polymerization reaction is catalyzed by the enzyme cellulose synthase (UDP-glucose:1,4- $\beta$ -D-glucosyltransferase; E.C. 2.4.1.12) which is present in the cytoplasmic membrane (Bureau and Brown 1987). The activity of this enzyme is regulated by bis-(3',5')-cyclic diguanylic acid (Ross 1987).

Purification of the cellulose synthase activity from membrane preparations has been accomplished by an entrapment procedure (Lin and Brown 1989) similar in some



-3-

aspects to one used in the purification of chitin synthase from yeast (Kang 1984). The molecular weight of the native enzyme in Triton X-100 solubilized preparations appears to be 490 Kd as determined by gel filtration (Lin and Brown 1989). Electron microscopy of the purified preparation shows doughnut-shaped particles indicating that the cellulose synthase may be organized as a tetramer or octamer (Lin and Brown 1989). Lithium dodecyl sulfate polyacrylamide gel electrophoresis of the purified preparation shows two major bands with molecular weights of 93 Kd and 83 Kd. The resistance of the cellulose synthase activity and of the 83 Kd polypeptide to trypsin treatment has suggested that the 83 Kd polypeptide is the active cellulose synthase (Lin and Brown 1989). The occurrence of these two polypeptides is always observed in purified cellulose synthase preparations of this bacterium, suggesting that they may be subunits of an oligomeric cellulose synthase complex.

Photoaffinity probes have been used with glucan synthases to identify these enzymes in a variety of different species, and with varying subunit molecular sizes. In red beets, a 57 Kd polypeptide has been shown to be the substrate-binding component of (1,3)- $\beta$ -glucan synthase (Wasserman 1989). In cotton fibers and in mung bean, a 50 Kd polypeptide appears to bind the substrate (Delmer and Solomon 1989). In *A. xylinum*, Mayer et al. (1989) suggested a 67 Kd polypeptide as the substrate-binding subunit and a 57 Kd polypeptide as the activator-binding subunit of a 420 Kd oligomeric cellulose synthase. These investigators suggested the presence of similarly sized peptides in other cellulose-producing organisms such as *Agrobacterium tumefaciens*, mung bean, wheat, pea and cotton on the basis of immunochemical analyses.

35

-4-

Genes involved in the synthesis of other exopolysaccharides in a number of bacteria have been cloned using the standard approach of genetic complementation [Easson (1987); Harding (1987); Calvin and Hanawalt (1988)]. Mutants of *A. xylinum* defective in the

production of cellulose complement of cellulose synthase have been identified in particular strains. Certain of these mutants have been shown to be deficient in the activity of UDPG-pyrophosphorylase, the enzyme required for the synthesis of the cellulose synthase substrate molecule, UDP-glucose. Complementation of these mutants by cloned fragments from *A. xylinum*-derived DNA has led to the isolation of the gene encoding UDPG-pyrophosphorylase (Valla 1989).

Recently, a purported operon for bacterial cellulose synthase was described in *A. xylinum* [Wong et al. 1990]. This operon has been suggested to consist of four genes in the order *bcsA*, *bcsB*, *bcsC*, and *bcsD* transcribed as a polycistronic mRNA. The second gene in the operon coding for polypeptide *bcsB* was suggested by these authors to be the cellulose synthase catalytic subunit gene, based on amino acid sequence of a 91 Kd polypeptide purified by product entrapment from trypsin treated membranes and supported by complementation analysis of mutants.

Surprisingly, however, no mutants actually deficient in cellulose synthase activity have been identified. This is despite the fact that there have been a large number of mutants identified which morphologically appear to be cellulose-deficient, but which synthesize small amounts of an altered crystalline polymorph of cellulose (cellulose II) and have the wild type level of cellulose synthase activity when assayed *in vitro* for cellulose synthesis [Saxena and Brown (1989); Roberts et al. (1989)].

-5-

Thus, it has been considerably difficult to apply the standard approaches of classical genetics or even the powerful techniques of molecular biology to the study of cellulose biosynthesis. Lacking purified quantities of the enzyme, lacking detailed information of the subunit architecture, lacking means to specifically identify the substrate-binding subunit, and lacking a simple genetic method of isolating mutants deficient in cellulose synthase activity, have each hampered the ability of researchers to isolate DNA segments encoding cellulose synthase. This is particularly true for the higher plants such as the important cellulose fiber-producing cotton species.

The present invention for the first time, provides the vectors, DNA segments, purified protein, antibodies, methods of cloning, and recombinant host cells, seeds and plants necessary to obtain and use a recombinant  $\beta$ -1,4 glucan synthase and related biological molecules. Thus, the difficulties encountered with applying the standard approaches of classical genetics or techniques of molecular biology to the study of cellulose biosynthesis have been overcome. Accordingly, the present invention concerns generally compositions and methods for the preparation of recombinant  $\beta$ -1,4 glucan synthase and related biological molecules of both prokaryotic and eukaryotic origin. Importantly, it is believed that the methods and compositions of matter of the invention may be applied to fiber-producing, agronomically important higher plants such as cotton as well as woody producing plants.

In certain general and overall embodiments, the invention concerns recombinant vectors and isolated DNA segments encoding a  $\beta$ -1,4 glucan synthase peptide or of a  $\beta$ -1,4 glucan synthase-related peptide. The DNA segments of the invention may encode biologically functional

-6-

equivalent protein or peptides which have variant amino acid sequences, such as with changes selected based on considerations such as the relative hydropathic score of the amino acids being exchanged.

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to the class of enzymes capable of polymerizing exopolysaccharides such as the enzyme cellulose synthase which is capable of polymerizing the exopolysaccharide cellulose. It is understood that in referring to a  $\beta$ -1,4 glucan synthase, the present application refers to holoenzyme which may, in some cases, include a multiple subunit molecule. In certain instances, the holoenzyme may be composed of multiple copies of the same polypeptide. In other cases, the holoenzyme may be composed of single or multiple copies of different polypeptides. For instance, in the case of the glucan synthase, cellulose synthase, the native holoenzyme when solubilized from membranes of A. xylinum appears to be a multiple subunit with a molecular weight of 490 Kd. These holoenzymes appear to be composed of multiple copies of at least two different polypeptides of 83 and 93 Kd molecular weights organized into tetramers or octamers. Therefore, when referring to the  $\beta$ -1,4 glucan synthase, cellulose synthase, it is understood to include both polypeptides. In some instances, the terms "glucan synthase" or "glucan synthase-related" are used inter-changeably with " $\beta$ -1,4 glucan synthase" or " $\beta$ -1,4 glucan synthase-related."

Where it is possible to distinguish the function of a given polypeptide, the function is identified by the modifier "catalytic" where a catalytic function can be ascribed to the polypeptide. Where catalytic function has not yet been ascribed to the polypeptide, but such peptide is known to be associated with glucan biosynthesis, the modifier "glucan synthase-related" is used.

-7-

More specifically, in the context of the present invention, the term  $\beta$ -1,4 glucan synthase is intended to refer to peptides or proteins having the biological and the immunological identity of the cellulose synthase of the cell lines enabled by the present invention. For example, such cell lines would include cells of Acetobacter xylinum. Generally, the A. xylinum cellulose synthase of the invention will refer to a 723 amino acid peptide or protein in that this is the precise length of the only presently sequenced cellulose synthase. However, the invention does not preclude and, in fact enables, preparation or use of shorter or longer peptides or proteins, so long as a peptide or protein has similar in kind biological activity and/or a cross reactive immunological reactivity, for example, as defined by rabbit polyclonal antisera. For instance, the other wild type strain of A. xylinum ATCC 23769 which was used by the present inventors possesses a 75 Kd polypeptide as a catalytic subunit of cellulose synthase by using the methods of the invention. Similarly, certain of the glucan synthase proteins of the cotton cells described herein may represent analogous proteins in the cotton fiber cell to the 83 Kd cellulose synthase of A. xylinum.

The term "cellulose synthase-related" is intended to refer to both DNA sequences, and peptides or proteins having the biological and the immunological identity of the cellulose synthase-related proteins of the cell lines enabled by the present invention. Therefore, the cellulose synthase-related proteins of the present invention would include the cellulose synthase catalytic subunit as well as any protein which co-purifies with the catalytic subunit in a tightly associated complex. In A. xylinum, such cellulose synthase-related proteins would include the 723 amino acid catalytic subunit as well as the tightly associated 809 amino acid polypeptide.

In addition, "cellulose synthase-related" may be used in conjunction with DNA sequences. Therefore, the cellulose synthase-related DNA sequences of the present invention would include DNA sequences encoding the  
5 cellulose synthase catalytic subunit as well as the DNA sequences encoding

~~cellulose synthase catalytic subunit in a tightly associated complex.~~  
Furthermore, once such DNA sequences are identified, the present invention enables one to identify further  
10 cellulose synthase-related proteins which are found to be contained in an operon, or other coordinately controlled genetic system, with the catalytic cellulose synthase protein.

15 In certain general aspects, the invention relates to the preparation and use of DNA segments, including vectors or DNA fragments, having a sequence encoding a  $\beta$ -1,4 glucan synthase polypeptide or related polypeptides. For vectors, any number are known in which DNA sequences of  
20 the invention may be incorporated. The vector pUC18 has been demonstrated to be of particular value. Likewise, the related vectors M13mp18 and M13mp19 may be used in certain embodiments of the invention, in particular, in performing dideoxy sequencing.

25 In certain embodiments, the vector will contain a substantially purified DNA fragment which encodes at least a useful portion of a cellulose synthase polypeptide which  
30 includes the amino acids 1 to 723 of Figure 1, or functionally equivalent amino acids. Alternatively, the vector will contain a substantially purified DNA fragment which encodes at least a useful portion of a cellulose synthase-related polypeptide which includes the amino  
35 acids 1 to 809 of Figure 13, or functionally equivalent amino acids. Recombinant vectors and isolated segments

may, therefore, variously include the basic  $\beta$ -1,4 glucan synthase or related molecule coding region itself or may contain coding regions bearing selected alterations or modifications in the basic coding region of  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related molecules. Alternatively, such vectors or fragments may encode larger proteins or peptides which nevertheless include the basic coding region or regions. The vectors or fragments may encode both the glucan synthase and glucan synthase-related proteins, or parts of such combinations. The vectors and fragments which encode combinations of glucan synthase and glucan synthase-related proteins may contain such combinations as they are organized in naturally occurring gene sequences (such as in operons) or such combinations may be organized differently from those encountered in naturally occurring gene sequences. In any event, it should be appreciated that due to codon redundancy, as well as biological functional equivalence, this aspect of the invention is not limited to the particular DNA sequences shown in Figures 1 or 13.

Recombinant vectors such as the foregoing are useful both as a means for preparing quantities of the  $\beta$ -1,4 glucan synthase-encoding DNA or  $\beta$ -1,4 glucan synthase-related protein-encoding DNAs themselves, and as a means for preparing the encoded protein and peptides. It is contemplated that where glucan synthase or glucan synthase-related proteins of the invention are made by recombinant means, one may employ either prokaryotic or eukaryotic expression and shuttle systems.

Prokaryotic host cells are disclosed in a preferred embodiment of the invention. However, in that prokaryotic systems are usually incapable of correctly processing eukaryotic precursor proteins, and since eukaryotic  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related proteins

are anticipated using the teachings of the disclosed invention, one may desire to express such sequences in eukaryotic hosts. However, even where the DNA segment encodes a eukaryotic glucan synthase or glucan synthase-related protein, it is contemplated that prokaryotic

Therefore, the invention can be used in combination with vectors which can shuttle between the eukaryotic and prokaryotic cells. Such a system is that of the Ti plasmids used in conjunction with the bacteria Agrobacterium tumefaciens.

Where expression of  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related protein in a eukaryotic host is contemplated, it most likely will be desirable to employ a vector such as a plasmid, that incorporates a eukaryotic origin of replication, such as those of the CaMV (cauliflower mosaic virus) and plasmids derived therefrom. Additionally, for the purposes of expression in eukaryotic systems, one will desire to position the glucan synthase or glucan synthase-related protein encoding sequence adjacent to and under the control of an effective eukaryotic promoter such as promoters used in combination with Ti plasmids. To bring a coding sequence under control of a promoter, whether it is eukaryotic or prokaryotic, what is generally needed is to position the 5' end of the translation initiation site of the proper translational reading frame of the protein between about 1 and about 50 nucleotides 3' of or "downstream" with respect to the promoter chosen. Furthermore, where eukaryotic expression is anticipated, one will typically desire to incorporate into the transcriptional unit which includes the glucan synthase or glucan synthase-related protein, an appropriate polyadenylation site. Typically, the polyadenylation site is placed about 30-2000



-11-

nucleotides "downstream" of the termination site of the protein at a position prior to transcription termination.

Accordingly, in certain preferred embodiments, the  
5 vectors of the invention are those where the  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related protein polypeptide encoding sequence is positioned adjacent to and under the control of an effective promoter. The  
10 vectors may be that set of vectors known well to those of skill in the art where the promoter comprises a prokaryotic promoter, the vector being adapted for expression in a prokaryotic host. Alternatively, the  
15 vectors may be those of common knowledge to skilled artisans where the promoter comprises a eukaryotic promoter, and the vector further includes a polyadenylation signal positioned 3' of the carboxy-terminal amino acid, and within a transcriptional unit of the encoded protein.

20 In certain embodiments of the invention, it is contemplated that DNA fragments both shorter and longer which incorporate sequences from Figure 1 or Figure 13 will find additional utilities, including uses as short  
25 DNA fragment hybridization probes, e.g., in screening both prokaryotic and eukaryotic recombinant clone banks. In any event, fragments including the sequence in Figure 1 or Figure 13 for stretches as short as 14 or so nucleotides, will generally find utility in accordance with these or  
30 other embodiments. By having stretches of at least about 14 nucleotides in common with the glucan synthase DNA sequence of Figure 1 or of the glucan synthase-related DNA sequence of Figure 13, or their complements, a DNA segment  
35 will typically have the ability to form a preferential hybridization with glucan synthase species DNA, particularly under more stringent conditions such as 0.15 M sodium chloride and 0.02 M sodium citrate, pH 7.4 at

-12-

about 50°C. While such a complementary or common stretch will typically ensure the ability to form a stable hybrid, longer stretches of complementary DNA may prove more desirable for certain embodiments. Thus, one may desire to use DNA segments incorporating longer stretches of  
[REDACTED]  
even 25 or 50 bases.

The invention also provides methods for isolating glucan synthase or cellulose synthase-related polypeptides from both recombinant and non-recombinant sources. Such a protein will typically include an amino acid sequence including to amino acids 1 to 723 of Figure 1 or including to amino acids 1 to 809 of Figure 13, respectively. However, the methods of the invention have been demonstrated to be successful in isolating a 75 Kd cellulose synthase polypeptide from *A. xylinum* strain ATCC 23769 as well as glucan synthase polypeptides of 66 Kd, 57 Kd and 35 Kd from cotton fiber cells. It will be apparent for instance, to those of skill in the art in light of the present specification that the 75 Kd polypeptide is different in molecular weight from the 83 Kd polypeptide described extensively herein. Furthermore, it will be obvious to such skilled artisans that the differences in molecular weights of these two polypeptides may result from actual differences in the primary structure of the amino acid chains themselves or may result from any number of post-translational modifications. Since such a protein represents the sequence for the catalytic subunit of the cellulose synthase protein, such a protein may be used directly to synthesize cellulose when provided the proper environment and substrate. Additionally, such a protein may be used to prepare an antibody for use in certain embodiments which antibody may be either a polyclonal or a monoclonal antibody and which, in any case, represents an

-13-

antibody immunologically reactive with any of the polypeptides of the invention.

5 The invention provides, therefore, a method of producing a recombinant  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related protein polypeptide. This method includes the use of a recombinant host cell where the recombinant host cell is capable of expressing a recombinant glucan synthase or glucan synthase-related  
10 protein polypeptide. Furthermore, the method for producing recombinant glucan synthase or glucan synthase-related protein provided in the invention includes culturing the host cell under conditions appropriate for expressing the polypeptide. Finally, the method of  
15 production claimed would include collecting the polypeptide thus expressed.

The method is particularly applicable where one desires to obtain a polypeptide corresponding to an 83 Kd catalytic subunit of cellulose synthase or of a cellulose  
20 synthase-related subunit such as the 93 Kd polypeptide of Acetobacter xylinum. However, it is proposed that the method may be directed to isolation of a glucan synthase polypeptide and a gene that is encoding a protein that is  
25 substantially similar to the 83 Kd catalytic subunit.

In particular embodiments, the method will typically involve selecting cells that are capable of glucan biosynthesis, particularly cells of Acetobacter xylinum  
30 and cotton fiber. A variety of cells are amenable to the method of the invention, for instance, cells of Agrobacterium, Rhizobium, Alcaligenes and particularly Sarcina. Of course, methods will typically involve culturing cells such that  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4  
35 glucan synthase-related protein is produced. In most cases, the cell lines used in the method will be one of

-14-

those cell lines which can actively engage in cellulose biosynthesis without addition of extraneous activators. Where cells of Acetobacter xylinum will be used, cultures will be grown in aerated and agitated culture medium containing Celluclast in order to free the cells trapped in the product cellulose.

as an inoculum (see U.S. Patent Application, Serial No. 022,904, filed March 6, 1987, Brown et al.).

In certain embodiments,  $\beta$ -1,4 glucan synthase may be partially purified from cells by solubilizing the cellular membranes. Partially purifying glucan synthase from cells by solubilizing the cellular membranes is preferably accomplished using digitonin. However, other solubilizing agents may be employed as long as the glucan synthase enzyme thus solubilized retains a substantial amount of its native activity. Other solubilizing agents which may be used include n-octyl glucoside and other nonionic detergents or Triton X-100.

Moreover, one may desire to even further purify the enzyme. If so, it has been found that a particularly useful approach employs "product entrapment". Product entrapment is explained in greater detail in the examples which follow. This technique entails generally layering solubilized enzyme and the substrate UDP-Glu solution on top of a cushion, e.g., of buffered glycerol, allowing biosynthesis of the  $\beta$ -1,4 glucan polymer to occur, centrifuging the resulting mixture and recovering the pellet which consists of  $\beta$ -1,4 glucan polymer along with the entrapped enzyme. This may even be followed by a series of chromatographic steps to still further purify the protein.

-15-

After obtaining a partially purified glucan synthase one may desire to admix it with a cellulose synthase activator in order to enhance the activity of the purified cellulose synthase. A useful cellulose synthase activator is bis-(3',5')-cyclic diguanylic acid in A. xylinum. In cotton fiber cells, bis-(2',3')-cyclic adenosine monophosphate is a useful activator. In addition, the present inventors have discovered that crude extracts of A. xylinum function as activators to achieve significant increases in azido-UDP-Glu binding in cotton fiber cells which fail to be enhanced significantly by purified cyclic-di-GMP. Thus, activators may be both well-characterized chemical substances or may represent complex and only roughly characterized substances as long as azido-UDP-Glu binding is enhanced in the target cell.

The present inventors have found a photoincorporation method to function in identifying the catalytic subunit of the  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related protein. Photolabeling may be desirable where other modes of labelling the active enzyme are insufficient. Photoincorporation of glucan synthase or cellulose synthase-related protein is accomplished most readily using a radioactively labeled azidonucleotide sugar analog capable of specifically interacting with the nucleotide sugar binding site of glucan synthase or glucan synthase-related protein. The photoincorporation technique is applicable to vascular plant cells like cotton fiber cells as well as to bacterial cells possessing glucan synthase or glucan synthase-related proteins.

Upon sequencing the purified  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related protein, one may construct oligonucleotide probes corresponding generally to some portion of the DNA segment which would encode such amino acid sequences as are determined from amino acid

-16-

sequencing the purified glucan synthase or glucan synthase-related protein. Oligonucleotide probes which incorporate particular DNA sequences will preferably correspond to at least a portion of the amino acid sequences of the various cellulose synthase or cellulose synthase-related protein polypeptides.

~~These polypeptides are synthesized by a variety of methods.~~

Preferably, these probes will be DNA sequences which encode portions of the Acetobacter xylinum 83 Kd cellulose synthase polypeptide or 93 Kd cellulose synthase-related polypeptide. In any case, the resulting probes will be used to probe a suitable source of DNA from a cell line capable of glucan biosynthesis.

The probing will usually be accomplished by hybridizing the oligonucleotide probes to a DNA source suspected of possessing a  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related protein gene. In some cases, the probes will constitute only a single probe, in others, the probes will constitute a collection of probes based on a certain amino acid sequence of the glucan synthase or glucan synthase-related protein and will account in their diversity for the redundancy inherent in the genetic code.

A suitable source of DNA from a cell line capable of glucan synthase or glucan synthase-related protein expression may be a genomic library of the cell line of interest. Alternatively, the suitable source of DNA may include total DNA from the cell line of interest. Once the hybridization process of the invention has identified a candidate DNA segment, one will desire to confirm that a positive clone has been obtained, e.g., by further hybridization, sequencing and/or expression and testing.

The invention also provides a means for obtaining a variety of recombinant host cells which incorporate a DNA

-17-

sequence in accordance with that depicted in Figure 1 or Figure 13. The host cell may be either prokaryotic or eukaryotic in nature. In any case, it is understood that the DNA segment encoding a glucan synthase or glucan synthase-related polypeptide will also possess the regulatory signals functional in the particular host cell. A preferred embodiment includes a recombinant plant entity which may comprise a plant cell (e.g., tissue cultured plant cells), a recombinant seed, or a recombinant plant having an incorporated gene encoding a glucan synthase or cellulose synthase-related polypeptide. The plant entity will possess the recombinant gene as a result of the in vitro introduction of the gene into a plant cell, wherein the recombinant gene is under the transcriptional control of regulatory signals functional in the particular plant entity. These regulatory signals will be appropriately selected to control the expression of the recombinant glucan synthase or glucan synthase-related protein in a manner to allow all requisite transcriptional and post-transcriptional modification.

Of particular interest in this regard will be plant entities which are cultivated for their content of glucan biopolymers such as cellulose and, most particularly, will be such plant entities as species of the genus Gossypium. Alternatively, more primitive entities such as algae or cyanobacterium are included within the description. Importantly, the gene encoding glucan synthase or glucan synthase-related protein may either be a gene which is heterologous to the plant cell, seed or plant in which the gene is introduced or it may be a copy of the gene homologous to that found in the plant entity being transformed. As used herein, the terms heterologous and homologous refer to the source of the recombinant DNA in reference to the DNA of the host cell. Thus, for instance, A. xylinum DNA from a particular strain of this

-18-

bacterium which is encoding a cellulose synthase or cellulose synthase-related polypeptide if used to transform another strain of cellulose synthase or cellulose synthase-related protein-producing bacteria or if used to transform a eukaryotic host cell would be the incorporation into either host cell's DNA. Conversely, the incorporation of the same A. xylinum DNA as above by retransforming an identical strain of A. xylinum would be the incorporation of a DNA segment homologous to that of the host cell's DNA.

In certain general aspects, then, a method of producing a glucan synthase or glucan synthase-related polypeptide is provided by the invention. First, one produces a recombinant host cell according to the methods and with the compositions of the invention such that the recombinant cell so produced is capable of expressing the polypeptide. Next, one cultures the host cell under conditions appropriate for expressing the polypeptide. Finally, according to the methods and with the compositions of the invention the recombinant polypeptide is recovered.

In other general aspects, a method of producing a  $\beta$ -1,4 glucan polymer is provided using the methods and compositions of the invention. Depending upon the particular glucan synthase one selects, one may direct the method to produce a given glucan polymer. For instance, if one desires a pure  $\beta$ -1,4 glucan polymer, one chooses a  $\beta$ -1,4 glucan synthase of the present invention such as the glucan synthase of Acetobacter. Of course, one may also desire to produce mixed glucan polymers in which case one may select a combination of glucan synthases of the invention. By way of example, if one desired to use the method to produce a  $\beta$ -1,4 glucan polymer, one first



-19-

obtains one of the recombinant host cells of the invention using the methods and compositions of the invention such that the host cell so obtained is capable of producing the  $\beta$ -1,4 glucan polymer. Next, one cultures the host cell  
5 under conditions appropriate for producing the  $\beta$ -1,4 glucan polymer. Finally, one recovers the  $\beta$ -1,4 glucan polymer thus produced.

It is also possible, using the methods and  
10 compositions of matter of the present invention to cause certain polymorphs of  $\beta$ -1,4 glucans to be exclusively or almost exclusively produced in vitro. By altering substrate concentrations made available to the enzyme preparations capable of producing the  $\beta$ -1,4 glucan, the  
15 present inventors can select, for instance, the nearly exclusive in vitro production of cellulose I polymorph from the enzyme derived from A. xylinum. This cellulose polymorph possesses many desirable characteristics such as the native cellulose I polymorph synthesized by living  
20 cells.

It will be obvious to those of skill in the art that the recombinant  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related polypeptide expressed by these host cells  
25 may use as its sole substrate UDP-glucose and produce a homopolymer such as cellulose. Alternatively, the substrate for the recombinant polypeptide may be other nucleotide sugars or mixtures of nucleotide sugars with UDP-glucose such as UDP-xylose and will result in  
30 heteropolymers. Furthermore, the recombinant glucan synthase or glucan synthase-related protein may act alone in these host cells or may act in concert with other enzymes to produce mixed polymer compositions.

35 Fig. 1. DNA/protein sequences of the A. xylinum cellulose synthase catalytic subunit gene. The coding

-20-

sequence begins at DNA position 636 and ends at DNA position 2804. The protein sequence begins at amino acid position 1 and ends at amino acid position 723. The single letter code used in the protein sequence here corresponds to amino acyl residues as follows: A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; Y, tyrosine; W, tryptophan; V, valine.

Fig. 2. 8% LDS-PAGE of cellulose synthase purified from Acetobacter xylinum. Lanes 1 to 5 were stained with Coomassie blue; lanes 6 to 8 were stained with silver. Lane 1, TS (20  $\mu$ g), Triton X-100-solubilized enzyme preparation; Lane 2, TSt (20  $\mu$ g), Triton X-100-solubilized enzyme preparation from trypsinized washed membranes; lane 3, TS-EP<sub>1</sub> (5  $\mu$ g), the purified enzyme by one step of entrapment, EP<sub>1</sub>, from the TS; lane 4, TSt-EP<sub>1</sub> (5  $\mu$ g), the enzyme purified by one step of entrapment, EP<sub>1</sub>, from TSt; lane 5, TSt-EP<sub>2</sub> (40  $\mu$ g), the enzyme purified by two steps of entrapment, EP<sub>2</sub>, from the TSt. This lane was overloaded deliberately in order to show the purity of the enzyme; lane 6, TSt-EP<sub>1</sub> (3.0  $\mu$ g); lane 7, TSt-EP<sub>2</sub> (p.5  $\mu$ g); lane 8, TSt-EP<sub>2</sub> (1.5  $\mu$ g).

Fig. 3. 8% LDS-PAGE of cellulose synthase in various purified fractions by using two detergents. Lane 1 to lane 4 were stained with Coomassie blue. Lane 1, OGS-EP<sub>1</sub> (10  $\mu$ g), the EP<sub>1</sub> purified from the n-octyl glucoside-solubilized enzyme preparation (OGS); lane 2, OGST-EP<sub>1</sub> (10  $\mu$ g), the EP<sub>1</sub> purified from OGSt; lane 3, DS-EP<sub>1</sub> (5  $\mu$ g), the EP<sub>1</sub> purified from digitonin-solubilized enzyme; lane 4, DS-EP<sub>1</sub> (10  $\mu$ g), the purified preparation from DS, but without 30% glycerol cushion during the entrapment.

-21-

Fig. 4. Subunit composition of the purified enzyme: effect of reduction and trypsin. (A) 8% LDS-PAGE of the EP<sub>1</sub> fraction from TS prepared from trypsinized washed membranes. Samples (10 µg protein per lane) were electrophoresed in sample buffer with mercaptoethanol (lane 1) and without it (lane 2). Note the prominence of the 93- and 83 Kd bands in lane 2. (B) 8% LDS-PAGE of the EP<sub>1</sub> fraction from DS in the absence of reducing agent. Samples (10 µg protein per lane) were gently shaken at 25°C in the presence of trypsin (trypsin/EP<sub>1</sub> = 4 µg/1,000 µg of protein concentration) for two minutes (lane 3), five minutes (lane 4), 10 minutes (lane 5) and 0 minutes (lane 6).

Fig. 5. Elution of cellulose synthase from a Con-A-Sepharose column. A 3 ml sample of the TS fraction was passed through a 7.5-ml Con A-Sepharose column which was then washed with enzyme buffer. The enzyme was eluted with 0.3 M methyl x-D-mannopyranoside in the enzyme buffer (arrow). Fractions of 1 ml were collected.

Fig. 6. Photographs of 8% LDS-PAGE without reducing agent, and stained either by the Schiff-periodate method using dansyl hydrazine (lane 1) or with a FITC-Con A (lane 2). Each lane contains 10 µg of the EP<sub>1</sub> purified from DS.

Fig. 7. Gel filtration elution pattern of TS and trypsin-treated TS on Sephacryl S-300. A 3 ml sample was applied. Fractions of 3 ml were collected. (A) elution profile for TS; (b) elution profile for TS which was treated with trypsin (5 µg/ml) at 25°C for 10 minutes.

Fig. 8. Photoaffinity labeling of cellulose synthase in various enzyme samples using 20 µM of [ $\beta$ -<sup>32</sup>P]5N<sub>3</sub>UDP-glc. Samples in lanes 1 and 2 are the membrane fractions, 40 µg of protein per lane. Samples in lanes 3 and 4 are the

digitonin-solubilized enzyme (20  $\mu$ g of protein per lane). Samples in lanes 5 and 6 are the purified cellulose synthase from digitonin-solubilized preparations (3  $\mu$ g of protein per lane). Either plus (+) or minus(-)  $\mu$ l of cellulose synthase activator contained in reaction mixture is indicated.

Fig. 9. Coomassie Blue stained 8% gel (A) and its corresponding autoradiography (B) of the cellulose synthase in purified fractions prepared by three detergents, digitonin, Triton X-100, and n-octyl glucoside, respectively. Samples in lanes 1 and 2 are the enzymes purified from the digitonin-extracted preparations (5  $\mu$ g of protein per lane). The sample in lane 3 is the enzyme purified from the n-octyl glucoside-extracted preparations (2.35  $\mu$ g of protein). The sample in lane 4 is the enzyme purified from the Triton X-100-extracted preparations (5  $\mu$ g of protein). The reaction mixture not illuminated with UV light is shown in lane 1.

Fig. 10. Autoradiograph demonstrating the prevention of [ $\beta$ - $^{32}$ P]5N<sub>3</sub>UDP-glc photoinsertion into the 83 Kd polypeptide by various concentrations of UDP-glc; 3  $\mu$ g of the enzyme purified from digitonin-extracted preparations for each lane. UDP-glc was added to reaction mixture at final concentrations of 0 (lane 1), 50 (lane 2), 100 (lane 3), and 300  $\mu$ M (lane 4).

Fig. 11. Autoradiograph demonstrating the saturation of [ $\beta$ - $^{32}$ P]5N<sub>3</sub>UDP-glc photoinsertion into the 83 Kd polypeptide. The final molar concentrations of [ $\beta$ - $^{32}$ P]5N<sub>3</sub>UDP-glc in the reaction mixtures were 20 (lanes 1 and 2), 50 (lane 3), 100 (lane 4), 300 (lane 5), and 500  $\mu$ M (lane 6), respectively. Use of preirradiated [ $\beta$ - $^{32}$ P]5N<sub>3</sub>UDP-glc is shown in lane 1.

-23-

Fig. 12. Autoradiograph demonstrating the labeling of a 57 Kd polypeptide in the digitonin-solubilized enzyme preparations. note that contaminating ( $^{32}\text{P}$ )glc-1-P may be present in the preparation of the [ $\beta$ - $^{32}\text{P}$ ]5N<sub>3</sub>UDP-glc probe. The digitonin-solubilized enzymes, 20  $\mu\text{g}$  of protein per lane, were incubated with 40  $\mu\text{M}$  of [ $\beta$ - $^{32}\text{P}$ ]5N<sub>3</sub>UDP-glc (4 mCi/ $\mu\text{mole}$ ) either alone, lanes 1 to 3, or in the presence of 500  $\mu\text{M}$  glc-6-P (lane 4), glc-1-P (lane 5), and glc-1,6-P (lane 6). For the control experiments, the preirradiated [ $\beta$ - $^{32}\text{P}$ ]5N<sub>3</sub>UDP-glc was used (lane 1) or the reaction mixture not irradiated as shown in lane 2.

Fig. 13. DNA sequence and deduced amino acid sequence of the 93 Kd polypeptide of *A. xylinum* cellulose synthase-related protein. The DNA sequence is numbered such that it is in continuation of the sequence in Figure 1. Numbers to the right of each line in the first column are the amino acid positions and in the second column are the nucleotide positions. The sequence coding for the cellulose synthase catalytic subunit stops at nucleotide position 2804 and the sequence for the 93 Kd polypeptide starts from position 2860. A potential ribosome-binding site for the 93 Kd polypeptide is marked by double lines under the sequence. The signal sequence (amino acid positions 1-16) is shown in italics. N-terminal amino acid sequence determined for the 93 Kd polypeptide and found to be matching with the deduced amino acid sequence (positions 27-40) is underlined. The transmembrane segment (amino acid positions 586-606) is indicated by dotted underline.

Fig. 14. Typical example from labeling experiments using azido-UD- $^{32}\text{P}$ -Glu of plasma membrane fractions from cotton fibers. Membranes from a 25 days post-anthesis ovary were used. Lane 1, no UV + activator; Lane 2, no activator + UV; Lane 3, crude GX activator from

Fig. 15. Negatively stained preparation of trypsin-  
10 treated/Triton-solubilized membrane fraction of  
Acetobacter which has been incubated in the presence of  
20mM UDP-glc, showing the cellulose synthase enzyme  
complexes (large arrows) and associated in vitro cellulose  
I product (small arrows).

In accordance with the present invention, access is provided to both recombinant and non-recombinant  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related protein. Methods are provided herein for the preparation of such enzymes in a substantially purified state. Since glucan polymers are ubiquitous throughout the plant kingdom, and since great effort has gone into attempts to isolate these highly important plant enzymes, the disclosure of this invention constitutes a true watershed in glucan polymer biochemistry.

The commercial applications of recombinant  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related protein and recombinantly produced glucan polymer have only begun recently to be explored since up until the present invention, no recombinant gene encoding a catalytic subunit of a glucan synthase or glucan synthase-related protein had been isolated. The present invention, provides such a capability for the first time. In preferred embodiments, the recombinant genes for cellulose synthase or cellulose synthase-related protein represented

-25-

by this invention are obtained using a microbial cell line. However, the present invention contemplates that recombinant genes for glucan synthase or glucan synthase-related protein may be derived from eukaryotic cell lines as well.

Commercial applications of microbially derived cellulose, from the bacterium Acetobacter xylinum for instance, has distinct advantages over traditional, non-microbial sources of this ubiquitous natural polymer. For instance, no delignification is required following harvest. Moreover, the product can be synthesized directly into an extremely strong non-woven textile of virtually any shape thereby reducing many steps in the conversion of natural cellulose into a finished product. The invention provides a recombinant cellulose synthase or cellulose synthase-related protein whereby the physical properties of the product of the enzyme (cellulose) such as crystallinity, hydrophobicity, and degree of polymerization can be controlled during synthesis. Most importantly, however, the present invention provides the unique possibility of producing commercially feasible levels of cellulose from a wide variety of substrates. It should be possible, using the recombinant gene of the invention, to transform, for instance, photosynthetic, nitrogen-fixing prokaryotes (cyanobacteria) or single-celled eukaryotes (algae).

The ability to alter physical characteristics of the cellulose in situ may provide an efficient method for producing a broad range of value-added products not possible before the recombinantly produced cellulose of the present invention was obtained. Previously, the development of a commercially feasible fermentation system for producing cellulose from bacteria has presented a significant technological challenge. Even using the most

-26-

preferred bacterial strain, large-scale fermentation has been complicated by a number of phenomena associated with the biology of acetic acid bacteria including strain instability, the synthesis of gluconic acid as a significant by-product, and poor oxygen diffusion through the cellulose product.

The present invention overcomes most of the complications of the prior art by providing a means for producing a recombinant  $\beta$ -1,4 glucan synthase or  $\beta$ -1,4 glucan synthase-related protein. The recombinant glucan synthase or cellulose synthase-related protein of the present invention allow production of a wide array of glucan polymer based products with significantly higher efficiency than the natural sources of cellulose. In so doing, the present invention may make its greatest contribution by preserving valuable natural resources.

For instance, cellulose from the cell walls of woody plants is formed in close association with lignin, hemicellulose, and other compounds. For many applications, such as in paper manufacture, wood cellulose must first be delignified by an extensive process known as pulping. Depending upon the process employed, pulping requires four or five processing steps following logging to obtain a partially purified cellulose material (Kirk-Othmer Concise Encyclopedia of Chemical Technology 1985). In contrast, Acetobacter xylinum and the recombinant cellulose synthase or cellulose synthase-related protein derived therefrom will be capable of synthesizing an extracellular ribbon of nearly pure cellulose directly into the incubation medium. The ribbons of individual bacterial cells interweave to form a hydrophilic cellulose membrane known as a pellicle. Alternatively, in vitro product synthesis of glucan polymers may be varied using



-27-

the methods of the invention depending on the glucan synthase selected or the substrates provided.

5       The production of relatively high yields of  
      Acetobacter cellulose without the corresponding synthesis  
      of lignin and hemicelluloses greatly facilitates the  
      process required to purify the cellulose. Nearly all of  
      the cellular biomass and incubation medium components can  
10       be efficiently removed from the pellicle by a simple  
      cleaning process. If necessary, the pellicle can then be  
      dried by various means as required for a particular end  
      product use. The simpler cleaning process of Acetobacter  
      cellulose may result in higher end product yields with  
15       greater purity than can be obtained by the pulping process  
      required for woody cellulose products.

      The native Acetobacter pellicle has outstanding  
      hydrophilicity and it is believed that the recombinant  
      cellulose synthase and cellulose synthase-related protein  
20       will share this attribute. Depending upon synthetic  
      conditions, it may have a water holding capacity ranging  
      from 60 to 700 times its dry weight. The hydrophilicity  
      of the cellulose pellicle is due in part to the extensive  
      interior surface area of the interstitial spaces of the  
25       "never dried" pellicle/microfibril matrix. The presence  
      of pore structures and "tunnels" within the wet pellicle  
      may be the reason for its rapid uptake of post synthetic  
      processing agents or dyes (Thompson 1988). Other sources  
      of cellulose, such as natural cotton and wood enter the  
30       manufacturing process in a more compacted form. This form  
      is not only less hydrophilic than microbial cellulose but  
      is also less susceptible to chemical treatments or to dye  
      uptake. As a consequence, harsher physical and chemical  
      processing is required, reducing cellulose yields and  
35       increasing processing costs. Recombinant sources of these  
      important enzymes will allow much improved products.

-28-

The strictly aerobic nature of Acetobacter may lend itself to industrial application. In undisturbed cultures, Acetobacter forms the cellulose pellicle at the air/liquid interface. Through the use of gas permeable molds, "microwoven" textiles of virtually any shape can be produced (U.S. Patent Application, Serial No. 220,800

~~U.S. Patent Application, Serial No. 220,800~~

684,844], filed April 17, 1989, Brown et al.). It has been demonstrated that a molded, seamless fabric in the shape of a glove can be formed *in situ* by an active Acetobacter culture. These microbial textiles can be synthesized into extremely thin, pliable forms which are gas and liquid permeable. The recombinant cellulose synthase and cellulose synthase-related protein of the present invention being derived from these bacterial cells will most likely share these unique advantages. Moreover, the significant advantage presented by the recombinant cellulose synthase and cellulose synthase-related protein will overcome the problems associated with the use of the natural bacterium.

The native Acetobacter pellicle has mechanical properties, including shape retention and tear resistance, which are superior to many synthetic fibers. It has been reported that following heat and pressure treatment, microbial cellulose has a Young's Modulus (a measure of shape retention) of 30 giga-Pascals, 4 times greater than any organic fiber (*The Japan Industrial Journal*, May 15, 1987; *Japanese New York Times*, May 29, 1987). The same report indicates that the tensile strength (tear resistance) of the treated material is five times greater than polyethylene or vinyl chloride films. Sony Corporation (Japan) has taken advantage of the shape retention properties of the treated Acetobacter cellulose in the development of a high fidelity audio speaker diaphragm made from the material (*The Japan Industrial*

-29-

Journal, May 15, 1987; Japanese New York Times, May 29, 1987). The Ajinomoto Company (Japan), a collaborator with Sony in the development of the cellulose treatment process, has begun distributing samples of ultrahigh strength paper manufactured from Acetobacter cellulose (The Japan Industrial Journal, May 15, 1987; Japanese New York Times, May 29, 1987). These commercial applications are indicative of the types of applications which will benefit significantly from the current inventions compositions and methods.

Perhaps the most significant advantage of the invention, however, stems from the possibility that it presents to produce cellulose from a wide array of potential substrates. Acetobacter xylinum, itself, is capable of producing cellulose from a variety of inexpensive carbon substrates. Widely available commodity sources of carbon which may be utilized by Acetobacter include dextrose (glucose), sucrose, fructose, invert sugar, ethanol and glycerol [Tarr and Hibbert (1934); Barsha and Hibbert (1934)]. This diversity of utilizable substrates provides considerable flexibility in the location of the manufacturing facility since at least one of these substrates is produced in virtually every region of the world. Flexibility in the manufacturing process is enhanced by the ability to substitute among a variety of possible fermentation substrates and provides some independence from impact of price increases for an individual substrate. The ability to employ a substrate diversification strategy is particularly advantageous in view of the significant price volatility associated with international agricultural commodity products.

In particular, with the methods and compositions of the present invention, an even wider range of potential substrates becomes possible. This wide range of

-30-

substrates is particularly advantageous when combined with the variable  $\beta$ -1,4 glucan synthases of the present invention. It is not unlikely that the recombinant genes disclosed herein when placed on the appropriate vector and into the appropriate cell line, may be capable of

directing  $\beta$ -1,4 glucan synthesis in a photosynthetic, nitrogen fixing cell thereby converting carbon dioxide and water directly into the desired product. The substantial advantages represented by this embodiment will be apparent to those of skill in the art.

A major advantage of the  $\beta$ -1,4 glucan biopolymer technology lies in the ability to control the physical characteristics of the native glucan biopolymer as it is being synthesized. These advantages will be even more available utilizing the recombinant glucan synthase or glucan synthase-related protein of the invention. Previous investigations have demonstrated that the addition of direct dyes such as Congo Red, fluorescent brightening agents such as Tinopal LPW, and carboxycellulose derivatives to the Acetobacter culture can alter the assembly of the cellulose ribbons [Ben-Hayyim and Ohad (1965); Haigler et al. (1982)].

Depending upon the agents employed and their concentration, a variety of cellulose structures can be produced. The variation in these structures can range from individual microfibrils that are loosely associated into ribbons to completely disassociated fibrillar structures, resulting in amorphous cellulose. The extension of this earlier research has resulted in the development of a proprietary process which can significantly effect the absorptive properties of Acetobacter cellulose. This process enables nearly complete rehydration of the pellicle to its original hydrophilicity even after three cycles of wetting followed

by drying at elevated temperatures (U.S. Patent Application, Serial No. 022,904, filed March 6, 1987, Brown et al.). It may be possible that Acetobacter cellulose synthesized by this process can be used as a reusable super-absorbent material. At any rate, the recombinant glucan synthase or glucan synthase-related protein of the present invention greatly enhances the already substantial benefits of these procedures.

10       The ability to control the glucan biosynthesis process within a closed system provides several advantages. It enables the manufacturer to alter the properties of the recombinant glucan such as cellulose to a greater degree than would be possible by post-synthetic  
15       processing of other sources of cellulose. By manipulating the cell density, incubation conditions, fermentation vessel configuration, and media components, the opacity, strength and pliability of the harvested cellulose product can also be controlled. It is possible to control the  
20       degree and type of polymerization of the cellulose as well (U.S. Patent 450,324, issued December 19, 1982).

      The compositions and methods of the invention which now make possible these commercially desirable goals, are  
25       exemplified below. In order to achieve the considerable advantages of the invention, purification of a  $\beta$ -1,4 glucan synthase and/or  $\beta$ -1,4 glucan synthase-related protein to a substantially purified state must first be accomplished. Next, the substantially pure glucan  
30       synthase or glucan synthase-related protein is used in order to identify the catalytic subunit of a multiple subunit enzyme. Following this identification, the catalytic subunit is at least partially sequenced and used to design oligonucleotide probes for hybridizing to the  
35       DNA of a glucan synthase containing cell line. The resulting hybridizing DNA is cloned and used to derive a

full length glucan synthase or glucan synthase-related protein gene from the DNA source.

5 In the case of cellulose synthase of A. xylinum, probes capable of identifying the cellulose synthase catalytic sequence were further useful in identifying the

[REDACTED]

10 It is anticipated that other such operonic associations may be encountered in other  $\beta$ -1,4 glucan synthase systems. Once these steps have been accomplished, the substantial advantages provided by the compositions of the invention including the substantially purified protein and the DNA segments may be used in a wide range of applications and

15 recombinant cellulose synthase or cellulose synthase-related protein and use of the DNA probes of the invention to probe for similar genes in other DNA sources including eukaryotic plant DNAs.

20 Summary of Purification Studies

Beyer et al. (1981) presented several problems that must be overcome if a glycosyltransferase is to be successfully purified. These included: (i) selection of

25 a cell strain which can produce a high yield of active transferase; (ii) an efficient harvest of viable cells from a selected culture method; (iii) a factor for stabilization of the enzyme; (iv) choice of the detergent; and, (v) an efficient purification procedure.

30 During the course of purification of cellulose synthase, the present inventors encountered a number of similar obstacles as well as ones unique to the glucan synthases. Several strategies had to be developed in

35 order to overcome these problems. First, an A. xylinum strain ATCC 53582 was selected since it has a greater

-33-

enzyme activity than ATCC 23769. Second, a method for production of high yields of essentially cellulose-free Acetobacter cells using cellulase was refined [Dillingham (1961); U.S. Patent Application, Serial No. 900,384, filed August 26, 1986, Brown et al.]. Third, Triton X-100 was selected as the detergent and glycerol was selected as the enzyme stabilizer.

The present inventors tested various purification methods, including sucrose or glycerol gradient ultracentrifugation, gel filtration with Sephacryl S-300, metal chelating affinity chromatography, anion-exchange chromatography with DEAE-Sephacryl CL-6B, and affinity chromatography with UDP-glucuronic acid-agarose. However, all either failed or resulted in only a 2-3 fold purification of the synthase.

A high degree of purification was achieved by in vitro cellulose-entrapment. The reason for such a high degree of purification being achieved is unclear. However, it could be due to: (a) the enzyme may be tightly enclosed by its insoluble product and thus coprecipitates as speculated by Kang et al. (1984) for chitin synthetase; (b) the density of cellulose is high, about 1.27-1.6 (*CRC Handbook of Chemistry and Physics*, 1984), which allows the enzyme product complex to pass through the 30% glycerol layer; and/or, (c) the 30% glycerol content of the cushion solution keeps most of the proteins in the reaction mixture during centrifugation.

It has been reported that three different subunits of the photosystem II reaction center could be isolated using Triton X-100, whereas five different subunits were obtained using digitonin (Nanba and Satoh 1987). Both purified preparations have the same photochemical activity. From these observations, the inventors proposed

-34-

that different detergents might solubilize an oligomeric enzyme complex in association either with or without non-essential proteins, even after purification. This may also be the case with the purification of cellulose synthase using different detergents. Under reducing conditions, the 83 Kd and 93 Kd bands were observed in the most purified preparations. Furthermore, the 93 Kd band was degraded and only the 83 Kd band remained if the purified preparation was treated with trypsin. These results, in combination with the evidence from the characterization of cellulose synthase as a glycosylated protein, suggested that the 83 Kd band is an essential part of the active cellulose synthase. However, the possible involvement of the 93 Kd polypeptide with the cellulose synthase could not be excluded since the enzyme may consist of non-identical subunits. In addition, the presence of the other protein bands in the most purified preparations was still a considerable concern. Definitive identification of the subunit composition of cellulose synthase had to await further investigation.

As shown in Fig. 4 (lane 1 and 2) which is described more fully hereafter, the 93 Kd band observed under reducing conditions and a 101 Kd band seen under non-reducing conditions could be the same polypeptide with intramolecular disulfide bonds. The presence of two small spots in the position of the 93 Kd band under non-reducing conditions (Fig. 4, lane 1, arrowheads), may be due to the partial degradation of intramolecular disulfide bonds during the preparation of the enzyme fraction. This also may explain the occurrence of two peaks present in the gel filtration elution profiles. In any case, such extraneous bands increased considerably the difficulty associated with the identification of the catalytic cellulose synthase polypeptide.



Dennis and Colvin (1965) noted that incubation of lysed cell preparations with trypsin at room temperature for 24 hours completely removed the cytoplasmic membrane and associated materials, and completely inhibited  
5 cellulose synthesis. They suggested that the site of synthesis might be the cytoplasmic membrane. Recently, Bureau and Brown (1987) also suggested that the cellulose synthase is located on the cytoplasmic membrane. However, their studies showed that cellulose synthase activity is  
10 maintained following brief trypsin treatment. Trypsin is well known for degrading proteins and also is effective for the cleavage of the peptidoglycan attachment region of lipoprotein in Gram-negative bacteria (Braun 1975).

15 In studies disclosed herein, trypsin was found to have no effect on cellulose synthase activity and resulted in more efficient detergent solubilization of the enzyme. However, it was not clear: (i) why trypsin degraded the solubilized form of the 93 Kd polypeptide, but did not  
20 degrade the membrane-bound form; or (ii) why the specific activity of the peak fraction from trypsin treated (TS; triton solubilized) is twice as great as the non-treated sample in gel filtration chromatographic studies. Elucidation of the interaction between trypsin and  
25 cellulose synthase required more study. Certainly, however, the ability to purify cellulose synthase provides a basis for certain surprising aspects of the invention. These techniques represent the first instance of the substantial purification of the cellulose synthase from A. xylinum, in particular, or any  $\beta$ -1,4 glucan synthase, in  
30 general.

Summary of the Studies Designed to Identify  
the Active Subunit of a Glucan Synthase

5 By using a photoaffinity labeling method, the  
substrate binding subunit of cellulose synthase, e.g.,  
from Acetobacter xylinum strain ATCC 53582, was shown to

the findings of others which identify an 85.3 Kd  
polypeptide, homologous to the 93 Kd polypeptide of the  
10 present invention, as the catalytic subunit. This 93 Kd  
polypeptide may include or comprise a regulatory subunit  
of cellulose synthase (i.e., the c-di-GMP binding  
subunit). Conversely, the 83 Kd polypeptide may be  
strictly a catalytic subunit with regulatory functions  
15 segregated into a distinct polypeptide, such as the 93 Kd  
polypeptide. In fact, there exists circumstantial  
evidence that the 93 Kd polypeptide is the effector-  
binding subunit (Mayer et al. 1989). Thus, the possible  
involvement of the 93 Kd polypeptide in the cellulose  
20 synthase complex cannot yet be excluded since the enzyme  
complex may consist of non-identical subunits for various  
functions in the polymerization and crystallization of  
cellulose (Lin and Brown 1989).

25 The 83 Kd polypeptide had been partially  
characterized as a glycoprotein by lectin affinity  
chromatography, Schiff-periodate and fluorescein  
isothiocyanate-Concanavalin A staining analyses (Lin and  
Brown 1989). However, since membrane associated  
30 glycoproteins have been widely found to be difficult to  
purify, the finding that [ $\beta$ -<sup>32</sup>P]5N<sub>3</sub>UDP-glc binds to the 83  
Kd catalytic subunit of cellulose synthase in A. xylinum  
provided the present inventors with a powerful tool for  
elucidation of the structure, function, and regulation of  
35 the cellulose synthase. Furthermore, it provided one  
means for the isolation of recombinant genes encoding the

-37-

5 catalytic 83 Kd polypeptide as well as the 93 Kd polypeptide immediately downstream. Only upon purification did it become feasible to sequence the polypeptide and construct an oligonucleotide probe for cloning.

10 It is also important to note here that the present inventors have applied the techniques of the invention to another wild type strain of A. xylinum known to produce cellulose I pellicles, ATCC 23769, and to a pellicle-free mutant derived from the ATCC 53582 strain (called NQ5). In so doing, the present inventors identified a 75-Kd polypeptide as the catalytic subunit of cellulose synthase which is in contrast to the 83-Kd subunit from ATCC 53582. 15 Thus, the techniques described herein, are generally applicable for identifying the catalytic subunit of purified cellulose synthases in various bacterial strains.

20 Furthermore, the azido probe techniques of the invention coupled with incorporation of radioactive UDP-Glu into glucan product have been demonstrated to be effective in identifying glucan synthases in the fiber-producing cells of cotton which make  $\beta$ -1,4 and  $\beta$ -1,3 glucan.

25

### Cloning and Sequencing Studies

30 Thus, the present invention provides a predictably successful, albeit difficult, method for the purification of glucan synthase made feasible by the techniques discussed above. Using solely prior art techniques, others had failed to obtain glucan synthase of adequate purity and quantity to enable either the identification of the active subunit or the sequencing of the active subunit polypeptide. 35 Furthermore, considerable disagreement exists in the art concerning the size and immunological

characteristics of the catalytic subunit of the cellulose synthase.

Application of cellulose entrapment, use of the  
5 cellulose synthase activator to enhance cellulose synthase  
activity, and photoincorporation identification of  
catalytic subunits, among other techniques, all combined to  
allow the present inventors to supercede the prior art and  
go on to sequence the glucan synthase polypeptides and,  
10 ultimately, to clone the genes encoding them. It will be  
appreciated by those of skill in the art that once  
provided with the techniques for obtaining a substantially  
purified glucan synthase, and with the amino acid  
sequences of the present invention, many of the difficult  
15 steps necessary for cloning and sequencing the gene  
encoding a glucan synthase polypeptide have been overcome  
by the present invention.

Armed with the highly purified catalytic subunit of  
20 cellulose synthase, the present inventors were poised to  
utilize the techniques of molecular biology to clone and  
sequence the genes associated with cellulose synthase.  
However, considerable difficulties were encountered even  
at this stage.

Initially, the inventors attempted to obtain the  
amino acid sequence of the purified enzyme and failed.  
Only an ambiguous sequence resulted from these studies.  
However, upon proteolytic treatment with trypsin, a  
30 relatively non-ambiguous sequence was obtained. Based on  
the unambiguous sequence of the invention, eight 17-mer  
oligonucleotide were prepared for use as probes.

These probes were each used independently to  
35 determine which probe exhibited strongest binding  
potential when tested against total DNA derived from A.

-39-

xylinum. This technique was a substantially high-risk experiment since even strains of E. coli not known to contain a cellulose synthase gene sequence were found to strongly bind to certain of the probes. Therefore, there was certainly no guarantee that any of these probes, even if one should demonstrate binding potential to A. xylinum, would be capable of hybridizing to the actual cellulose synthase DNA.

In fact, a further complication arose from the very demonstration that E. coli strains known to those of skill in the art readily bound the probes of the invention. Since these probes would most likely not be capable of detecting a recombinant gene sequence in such a background, the inventors were forced to forgo the typical technique applied by those of skill in the art, that technique being the creation of a total genomic DNA library based on the genomic DNA from A. xylinum. Instead, the inventors were forced to rely on the much less certain technique of using the selected probe to identify bands of hybridizing DNA from a gel containing total genomic A. xylinum DNA which had been treated with restriction endonuclease.

This only allowed the isolation of a certain subset of DNA which might hybridize specifically with the probe, so the resulting DNA was subcloned into a vector and used to transform host bacteria. Even further difficulties were encountered at this point since only very few recombinants were obtained, most likely due to the restriction of the A. xylinum heterologous DNA in E. coli. Additionally, since it was not possible to screen the host bacteria due to a high background of probe binding even under stringent conditions, the recombinant plasmids had to be reisolated from the clones, pooled into 50 isolates per group and tested once again with the probe DNA for

groups which demonstrated binding. Only after the application of these multiple-step techniques were the inventors herein able to isolate the recombinant plasmid which contained the hybridizing DNA, to subclone individual fragments of this DNA to the point that they contained substantially only the sequence encoding

resulting fragments were utilized to obtain a DNA sequence of 2912 base pairs which was a region of the DNA sequence encoding a protein consisting of 723 amino acid residues.

As noted previously, it is believed that modification and changes may be made in the structure of the recombinant glucan synthase protein and still obtain a molecule having like or otherwise desirable characteristics. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with complementary structures such as antigen-binding regions of antibodies (e.g., binding sites on receptor molecules). Since it is the interactive capacity and nature of a protein that defines that proteins biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence (or, of course, its underlying DNA coding sequence) and, nevertheless, obtain a protein with like or even countervailing properties (e.g., antagonistic v. agonistic). It is thus contemplated by the present inventors that various changes may be made in the sequence of the peptides (or underlying DNA) without appreciable loss of their biological utility or activity.

#### Biological Functional Equivalency

The importance of the hydropathic index of amino acids in conferring interactive biologic function on a

-41-

protein has been discussed generally by Kyte and Doolittle (1982) wherein it is found that certain amino acids may be substituted for other amino acids having a similar hydrophathic index or score and still retain a similar biological activity. As displayed in Table I below, amino acids are assigned a hydrophathic index on the basis of their hydrophobicity and charge characteristics. It is believed that the relative hydrophathic character of the amino acid determines the secondary structure of the resultant protein, which in turn defines the interaction of the protein with substrate molecules. It is proposed that functional equivalency may typically be maintained where amino acids having no more than a  $\pm 1$  to 2 difference in index, and more preferably within about a  $\pm 1$  difference, are exchanged.

TABLE I

	<u>Amino Acid</u>	<u>Hydrophathic Index</u>
20	Isoleucine (Ile)	4.5
	Valine (Val)	4.2
	Leucine (Leu)	3.8
	Phenylalanine (Phe)	2.8
	Cysteine/cystine (Cys)	2.5
25	Methionine (Met)	1.9
	Alanine (Ala)	1.8
	Glycine (Gly)	-0.4
	Threonine (Thr)	-0.7
	Tryptophan (Trp)	-0.9
30	Serine (Ser)	-0.8
	Tyrosine (Tyr)	-1.3
	Proline (Pro)	-1.6
	Histidine (His)	-3.2
	Glutamic Acid (Glu)	-3.5
35	Glutamine (Gln)	-3.5
	Aspartic Acid (Asp)	-3.5

-42-

Asparagine (Asn)	-3.5
Lysine (Lys)	-3.9
Arginine (Arg)	-4.5

5           Thus, for example, isoleucine, which has a  
 hydropathic index of +4.5, can be substituted for valine  
 having similar biologic activity. Alternatively, at the  
 other end of the scale, lysine (-3.9) can be substituted  
 10 for arginine (-4.5), and so on.

Accordingly, these amino acid substitutions are  
 generally based on the relative similarity of R-group  
 substitutents, for example, in terms of size,  
 15 electrophilic character, charge, and the like. In  
 general, exemplary substitutions which take various of the  
 foregoing characteristics into consideration include the  
 following:

20

TABLE II

	<u>Original Residue</u>	<u>Exemplary Substitutions</u>
	Ala	gly; ser
	Arg	lys
25	Asn	gln; his
	Asp	glu
	Cys	ser
	Gln	asn
	Glu	asp
30	Gly	ala
	His	asn; gln
	Ile	leu; val
	Leu	ile; val
	Lys	arg
35	Met	met; leu; tyr
	Ser	thr



-43-

Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

5

### Site-Specific Mutagenesis

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, derived from the cellulose synthase sequence, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art as exemplified by publications such as Adelman, et al. (1983), incorporated herein by reference. As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage, for example, as disclosed by Messing et al. (1981), incorporated herein by

-44-

reference. These phage are readily commercially available and their use is generally well known to those skilled in the art.

5 In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded  
[REDACTED] a DNA sequence which encodes the glucan synthase sequence. An oligonucleotide primer bearing the desired mutated  
10 sequence is prepared, generally synthetically, for example by the methods described herein. This primer is then annealed with the singled-stranded vector, and subjected to DNA polymerizing enzymes such as E. coli polymerase I Klenow fragment, in order to complete the synthesis of the  
15 mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells such as E. coli cells and clones are  
20 selected which include recombinant vectors bearing the mutated sequence arrangement.

#### Host Cell Cultures and Vectors

25 In general, of course, prokaryotes are preferred for the initial cloning of DNA sequences and constructing the vectors useful in the invention. For example, E. coli. HB101 has been shown to be particularly useful. Other microbial strains which may be used include E. coli  
30 strains such as E. coli B, and E. coli X 1776 (ATCC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes may also be used for expression. The  
35 aforementioned strains, as well as E. coli W3110 (F-, lambda-prototrophic bacilli such as Bacillus subtilis, or

-45-

other enterobacteriaceae such as Salmonella typhimurium or Serratia marcesans, and various Pseudomonas species may be used.

5           In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of  
10       providing phenotypic selection in transformed cells. For example, E. coli is typically transformed using pBR322, a plasmid derived from an E. coli species and well known to those of skill in the art. The vector pBR322 contains genes for ampicillin and tetracycline resistance and thus  
15       provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins.

20           Those promoters most commonly used in recombinant DNA construction include the  $\beta$ -lactamase (penicillinase) and lactose promoter systems and a tryptophan (trp) promoter system each of which is well known to those of skill in  
25       the art. While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors.

30           In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used. Saccharomyces cerevisiae, the Agroor common baker's yeast is the most commonly used among eukaryotic microorganisms, although a  
35       number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for

-46-

example, is commonly used. This plasmid already contains the trp1 gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1. The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective selection for successful transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes [Hess et al. (1968); Holland et al. (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing a yeast-compatible promoter, origin of replication and termination sequences is suitable.

#### Cotton Transformation

An example is provided which illustrates the manner in which an agronomic or other eukaryotic plant species

may be transformed with recombinant  $\beta$ -1,4 glucan synthase. It is based on the successful transformation of cotton (Gossypium hirsutum) with a recombinant gene for kanamycin resistance from bacteria, a situation not unlike that  
5 which the present invention now makes possible for transformation of plant cells with, for instance, bacterial cellulose synthase. It will be recognized by those of skill in the art, that there are certain limitations unique to the glucan synthases of the present  
10 invention which may not be addressed in the example below. However, the example demonstrates that there are not insurmountable obstacles to transforming a cotton plant with a glucan synthase such as a bacterial cellulose synthase.

15 The technique basically utilizes a binary plant vector strategy based on the Agrobacterium tumefaciens Ti plasmid. It further involves the cloning of the glucan synthase gene in a plant transformation vector (PTV).  
20 This modified vector is then mated from E. coli into the Agrobacterium and recombinant colonies are selected which contain the recombinant gene. The Agrobacterium strain carries a "disarmed" Ti-plasmid. Virulence functions on the Ti-plasmid interact in trans with the border sequence  
25 on the PTV thereby mobilizing the glucan synthase gene into the plant cell and inserting the gene into one of the host plant's chromosomes. Callus derived from these transformed cotyledons is treated with various plant hormones and stimulated to regenerate whole recombinant  
30 plants according to techniques known to those of skill in the art.

There are, of course, a variety of methods known to those of skill in the art for introducing recombinant  
35 genes into plants as well as a number of plant species into which recombinant DNA has been introduced (see

-48-

generally, Gasser and Fraley 1989). In those systems where Agrobacterium-mediated transformation is efficient, such as in cotton as noted herein, it is the method of choice because of the ease and defined nature of the gene transfer system utilized. However, few monocotyledonous plants appear to be hosts for Agrobacterium, thus

delivery systems for recombinant DNA into these species.

Certain of these techniques rely on physical means of introducing DNA into the plant cell. Transformation of protoplasts has been achieved through facilitating DNA uptake by calcium phosphate precipitation, polyethylene glycol treatment, electroporation, or combinations of these methods. These techniques rely on introduction of recombinant DNA into protoplasts of the plant cells. Where regeneration of protoplasts has been shown to be difficult, mechanical means to introduce recombinant DNA into intact cells or tissues has been successfully used. In particular, the use of particle guns (high-velocity microprojectile technology) has been shown to affect transformation of plant cells. Other methods with the potential to achieve plant cell transformation with recombinant DNA include transfer into pollen, direct injection into reproductive organs, microinjection into cells of immature embryos and rehydration of dessicated embryos.

Furthermore, a growing number of species have been reported from which the production of transgenic plants has been achieved with one or another of the techniques noted above. The following is a recently updated list of such plants and a reference to the method used to affect the transformation (see generally, Gasser and Fraley 1989: Abbreviations; AT, Agrobacterium tumefaciens; AR Agrobacterium rhizog nes; FP, free DNA introduction into

-49-

protoplasts; PG, particle gun; MI, microinjection; IR, injection of reproductive organs).

	<u>PLANT SPECIES</u>	<u>METHOD</u>
5	<i>Herbacious Dicots</i>	
	Petunia	AT
	Tomato	AT
	Potato	AT
	Tobacco	AT, FP, PG
10	<i>Arabidopsis</i>	AT
	Lettuce	AT
	Sunflower	AT
	Oilseed Rape	AT, MI
	Flax	AT
15	Cotton	AT
	Sugarbeet	AT
	Celery	AT
	Soybean	AT, PG
	Alfalfa	AT
20	<i>Medicago varia</i>	AT
	Lotus	AT
	<i>Vigna aconitifolia</i>	FP
	Cucumber	AR
	Carrot	AR
25	Cauliflower	AR
	Horseradish	AR
	Morning Glory	AR
	<i>Woody Dicots</i>	
30	Poplar	AT
	Walnut	AT
	Apple	AT
35	<i>Monocots</i>	
	Asparagus	AT
	Rice	FP
	Corn	FP
40	Orchard Grass	FP
	Rye	IR

#### 45 Plant DNA Hybridization

Another example is provided to illustrate a proposed use of the recombinant glucan synthase of the present invention to probe plant DNA sources for hybridizing sequences. This method allows for a rapid microscale isolation of plant DNA without the use of CsCl gradients.

The technique was developed for Zea mays but has been successfully applied to a variety of other plant species including Nicotiana tabacum, N. plumbaginifolium, N. sylvestris, Lyscopericum sp., Amaranthus sp., Glycine max, and Petunia hybrida.

plant tissue DNA from leaf tissue by disrupting the cells of the leaf frozen in liquid nitrogen. The disrupted cell debris is then solubilized in detergent and buffer in order to remove most of the proteinaceous and polysaccharide materials. After filtering the debris from the buffered solution, plant DNA is then precipitated with alcohol, dried and resuspended in a buffered solution, then precipitated using a high salt-alcohol method. The resulting DNA may be used with restriction endonucleases and other DNA-modifying enzymes. It has also been demonstrated to be useful in filter hybridization using DNA probes such as those of the present invention.

As mentioned, in certain aspects, the DNA sequence information provided by the invention allows for the preparation of relatively short DNA (or RNA) sequences having the ability to specifically hybridize to gene sequences of the glucan synthase gene. In these aspects, nucleic acid probes of an appropriate length are prepared based on a consideration of the sequence shown in Figure 1 or Figure 13. The ability of such nucleic acid probes to specifically hybridize to the glucan synthase gene sequences lend them particular utility in a variety of embodiments. Most importantly, the probes can be used in a variety of assays for detecting the presence of complementary sequences in a given sample such as the plant DNA samples described here. However, other uses are envisioned, including the use of the sequence information for the preparation of mutant species primers, or primers



-51-

for use in preparing other genetic constructions such as for obtaining full length clones of sequences downstream or upstream of the original clones of the invention.

5           To provide certain of the advantages in accordance with the invention, the preferred nucleic acid sequence employed for hybridization studies or assays includes sequences that are complementary to at least a 14 to 25, or so, nucleotide stretch of the sequence shown in Figure 1 or Figure 13. A size of at least 14 nucleotides in  
10           length helps to ensure that the fragment will be of sufficient length to form a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches greater than 10 bases in length  
15           are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches  
20           of 14 to 20 nucleotides, or even longer where desired. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR technology of U.S. Patent 4,603,102, or by  
25           introducing selected sequences into recombinant vectors for recombinant production.

          Certain nucleotide sequences of the invention will be important for their ability to selectively form duplex  
30           molecules with complementary stretches of the gene. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degree of selectivity of the probe toward the target sequence. For applications requiring a high degree  
35           of selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, for

-52-

example, one will select relatively low salt and/or high temperature conditions, such as provided by 0.02M-0.15M NaCl at temperatures of 50°C to 70°C. These conditions are particularly selective, and tolerate little, if any, mismatch between the probe and the template or target strand.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate cellulose synthase coding sequences for related species, functional equivalents, or the like, less stringent hybridization conditions are called for in order to allow formation of the heteroduplex. In these circumstances, one would desire to employ conditions such as 0.15M-0.9M salt, at temperatures ranging from 20°C to 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In certain embodiments, it will be advantageous to employ nucleic acid sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal.

-53-

In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe, etc.). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label.

While it is not specifically addressed in this example, the use of the methods and the compositions of the present invention may be applicable in certain instances to non-plant eukaryotes. In particular, oriented cellulose has been shown to be a component of certain mammalian tissues. There is considerable evidence that polysaccharides play an important role in the stabilization of connective tissue fibers and as such may play a role in certain disease conditions in humans such as scleroderma conditions [see generally, Hall et al., *Proc. Royal Soc. B* 151; 497 (1960)].

In any event or from whatever source, once the DNA fragments have been detected via these techniques, standard approaches known well to those of skill in the art may be utilized to further isolate and clone the fragments. As alternative sources of recombinant glucan synthases, these new fragments will share all the

utilities of the compositions described specifically herein.

### Transformation of Cyanobacteria with Cellulose Synthase

5

~~Cyanobacteria (blue-green algae) are prokaryotes. They are unicellular organisms that have two things in common: they~~

are prokaryotes and they carry out oxygenic, plantlike photosynthesis. Many strains of cyanobacteria are also  
10 capable of fixing atmospheric nitrogen. These properties make cyanobacteria prime candidates for transformation with recombinant glucan synthase genes from A. xylinum in order to develop commercial scale glucan biopolymer production in a prokaryotic species capable of  
15 photosynthesis and nitrogen fixation. This is particularly attractive with regard to cellulose biosynthesis.

Typically, no cellulose biosynthesis has been  
20 demonstrated in cyanobacteria under normal growth conditions. However, cellulose produced in the form of a slime has been reported in Nostoc [A. Frey-Wyssling, The Plant Cell Wall, p.227, Gebruder Bornträger, Berlin (1976)]. The transfer of an A. xylinum cellulose synthase  
25 gene into cyanobacteria is made possible with the methods and compositions of the present invention.

Gene transfer has been established and host-vector systems have been developed for gene cloning in  
30 Cyanobacteria (Kuhlemeier and Van Arkel 1987). Two strategies have been employed for gene cloning in Cyanobacteria. One uses shuttle plasmid vectors that carry both a cyanobacterial and an E. coli replicon, each of which functions in its indigenous host. Depending on  
35 the sequence of the cloned gene, the selection applied, and the cyanobacterial host, the cloned gene can be

-55-

maintained on the autonomous plasmid or can be integrated into the cyanobacterial chromosome.

5       The other strategy exploits the efficient  
recombination system of certain cyanobacterial strains  
(Anacystis nidulans R2, Synechocystis sp. 6803) for a  
directed integration of the cloned gene into the  
chromosome (Golden et al. 1987). In the latter strategy a  
10       DNA vector that lacks a cyanobacterial replicon, but  
carries similar chromosomal sequences, is used. According  
to the imposed selective conditions, the cloned gene,  
either alone or together with the vector sequences,  
integrates into the chromosome.

15       Gene transfer in cyanobacteria is known to take place  
by conjugation and transduction as well as by  
transformation (Herdman 1982). Up to now, however, the  
only system of practical importance has been the  
transformation system (Porter 1988) and that is the  
20       preferred method described herein.

      A number of bacterial antibiotic resistance genes are  
expressed in cyanobacteria, where they are used for  
selecting transformed cells (Golden et al. 1987). Apart  
25       from these, the larvicidal gene of Bacillus sphearicus  
1593M was transformed into the cyanobacterium Anacystis  
nidulans R2, where it exhibited expression and toxin  
activity comparable to that observed with E. coli carrying  
the respective recombinant plasmids (Tandeau de Marsac et  
30       al. 1987). Use has also been made of the coding sequences  
of cat, lacZ, and lux to function as reporter genes in  
determining efficient promoter sequences (Friedberg 1988).  
The gene products of these genes can be assayed in  
cyanobacteria allowing scoring or selection of cells  
35       expressing the gene.

**EXAMPLE I:      PURIFICATION OF CELLULOSE SYNTHASE**

Materials - Reagents were obtained from the following sources: UDP-<sup>14</sup>C]glc, lithium dodecyl sulfate, ICN;  
5 polyethylene glycol (PEG)-3350, Triton X-100, n-octyl glucoside, trypsin (EC 3.4.21.4), ~~trypsin inhibitor~~

~~mannopyranoside, dansyl hydrazine, fluorescein isothiocyanate-labeled Con A (FITC-Con A), Sigma;~~  
10 digitonin, Serva; Celluclast, Novo Enzymes; guanosine-5'-triphosphate (GTP) dilithium salt, Boehringer Mannheim. All operations were performed at 0-5°C, unless otherwise indicated.

15 Cells - Two bacterial strains of A. xylinum, ATCC 53582 and ATCC 23769, were used. Cultures were maintained on Schramm and Hestrin's glucose medium as previously described (Schramm and Hestrin 1954). A single colony, grown for 5-7 days at 28°C on agar plates, was transferred  
20 to a flask containing 200 ml of culture medium and 75 µl filtered Celluclast, and cultured at 28°C on a rotary shaker set at 120 rpm for 42-48 hour [Dillingham (1961); U.S. Patent application No. 900,384, filed August 26, 1986]. A 5 ml aliquot of the starting culture was then  
25 transferred to a new flask having the same amount of medium and Celluclast and cultured under the same conditions. After 48 hours, the cell suspension was passed through 8 layers of cheesecloth and then centrifuged at 10,000 x g for 10 minutes. The pellets  
30 were washed once in a buffer of 50 mM Tris-10mM MgCl<sub>2</sub>-1 mM EDTA, pH 7.5 (TME) as described by Glaser (Glaser 1958). The yield was about 1 gm of cells, dry weight, per liter of culture. The washed cell pellets were resuspended in TME containing 20% (weight/ volume) PEG-3350.

35

Preparation of Membranes and Activator - "Washed membranes" and "crude factor" were prepared as described previously (Ross 1986) except that the cells were disrupted in an Aminco French pressure cell at 16,000 lb/in<sup>2</sup>. Trypsinized membranes were prepared as follows: to 10 ml of washed membranes (protein concentration, 5 mg/ml), 0.2 mg of trypsin was added and gently shaken at 25°C for 30 minutes. After the addition of 0.2 mg of trypsin inhibitor for 20 minutes, the mixture was centrifuged at 18,000 x g for 20 minutes and washed once in TME.

Cyclic diguanylic acid, an activator of the cellulose synthase (Lin et al. 1985), was obtained in two ways as described (Ross 1986), but with several modifications: (i) 8 ml of crude factor was mixed with 1 ml 10 mM GTP, 0.1 ml 100 mM CaCl<sub>2</sub> and 0.9 ml TME buffer, and incubated at 37°C for two hours. The reaction was terminated by heating in boiling water for three minutes, followed by centrifugation at 2,000 x g for 10 minutes. This supernatant was called "crude Gx"; (ii) the activator was prepared as in (i) except that the reaction was terminated by addition of 0.6 M HClO<sub>4</sub> instead of heating. After centrifugation, the supernatant was neutralized with 5 M KOH and recentrifuged at room temperature. This supernatant was designated "N-Gx."

Solubilization by Nonionic Detergent - Either the washed membranes or the trypsinized membranes were resuspended in TME containing 20% glycerol, 0.6% (weight/volume) Triton X-100 to give a final protein concentration of 5.0 mg of membrane protein per ml. After homogenization with a Teflon homogenizer, the suspension was gently shaken for one hour, followed by centrifugation at 100,000 x g for one hour. The supernatant represented the Triton-solubilized enzyme which was designated "TS" if the washed

-58-

membranes were used, and "TSt" if the trypsinized membranes were used. The n-octyl glucoside-solubilized enzyme (OGS and OGSt are equivalent to TS and TSt) was prepared as TS except that 1% (weight/volume) n-octyl glucoside was used with a two hour shaking time. The digitonin-solubilized enzyme (DS) was prepared as

Cellulose Entrapment Method - The entrapment method used was described earlier (Kang 1984), with the following modifications: cellulose nitrate tubes, 9/16 x 3 3/4 in., were used. A 2.6 ml of cushion solution, consisting of TME buffer and 30% glycerol (v/v), were added to the bottom of the tubes. Subsequently, a mixture of 6.8 ml of the solubilized enzyme, 0.2 ml of 100 mM UDP-glucose and 3.4 ml of N-Gx, was layered on the top of cushion solution. The tubes were incubated in a 30°C water bath for 11 minutes, then were placed on ice for two hours, and centrifuged for 20 minutes in a Beckman SW 40 Ti rotor at 50,000 x g. After overnight storage at -80°C, each pellet was resuspended in extraction buffer to 1/30 the original loading volume and homogenized with a Teflon homogenizer. Extraction buffer was prepared with 0.1% detergent in TME buffer containing 20% glycerol. The suspension was centrifuged for 10 minutes at 15,000 x g. The supernatant was designated ES<sub>1</sub> and the pellet, EP<sub>1</sub>. The EP<sub>1</sub> was resuspended in extraction buffer to the same volume as ES<sub>1</sub>.

ES<sub>2</sub> and EP<sub>2</sub> were the resulting supernatant and pellet respectively, from a second entrapment step of ES<sub>1</sub>. All purified enzymes were stored at -80°C.

Con A-Sepharose Chromatography - Triton X-100 solubilized enzyme preparation, TS, was applied at a flow rate of one column volume/hour to a column (1 x 10 cm) of Con A-



-59-

Sephacryl 4B previously equilibrated in an "enzyme buffer" of TME, 1 mM  $\text{MnCl}_2$ , 1mM  $\text{CaCl}_2$ , 20% glycerol and 0.1% Triton X-100. The flow was stopped after the entire sample was applied and allowed to stand for one hour. The column was washed with 25 ml of enzyme buffer, and the bound protein was then eluted with 0.3 M methyl alpha-D-manno-pyranoside in enzyme buffer. One ml fractions were collected for enzyme assay.

Sephacryl S-300 Chromatography - A column (1.6 x 85 cm) of Sephacryl S-300 was equilibrated with TME buffer containing 0.1% Triton X-100 and 20% glycerol. The enzyme preparation, TS, was applied and eluted with the same buffer, at a flow rate of 11.0 ml/hr. Fractions of 3 ml were collected and assayed for enzyme activity. The column was calibrated with a series of molecular weight markers (Pharmacia), usually Blue Dextran 2000, thyroglobulin, ferritin, catalase, aldolase and albumin.

Enzyme Assay - Assays contained a final volume of 0.2 ml: 20  $\mu\text{l}$  of 0.5 M Tris-HCl (pH 9.6) containing 50 mM  $\text{MgCl}_2$  and 5 mM EDTA, 20  $\mu\text{l}$  of 0.1 M  $\text{MgCl}_2$ , 20  $\mu\text{l}$  of 10 mM  $\text{CaCl}_2$ , 25  $\mu\text{l}$  of TME buffer, 25  $\mu\text{l}$  of  $\text{H}_2\text{O}$ , 20  $\mu\text{l}$  of crude Gx, and 50  $\mu\text{l}$  of enzyme sample. Incubation was carried out in a 30°C water bath for either (a) 5 minutes in the final concentration of 20  $\mu\text{M}$  (110 cpm/pmol) UDP-[ $^{14}\text{C}$ ]glc or (b) 30 minutes in the final concentration of 1 mM (2.2 cpm/pmol) UDP-[ $^{14}\text{C}$ ]glc. The reaction was stopped with 2 ml of 0.5 M NaOH, and the radioactivity in alkali-insoluble materials was counted after filtration through glass-fiber filters, as described in detail (Bureau and Brown 1987).

Protein D termination - Protein concentrations were determined according to the method of Bradford (1976), using the protein assay kit of Bio-Rad Laboratories. A

-60-

modification of the Lowry procedure (Markwell 1978) was used only with membrane preparations. In both cases, bovine serum albumin was used as a standard.

5     Polyacrylamide Gel Electrophoresis - The samples were dissolved in sample buffer containing 2% lithium dodecyl

mercaptoethanol and 20% glycerol with 0.02% bromophenol blue. The incubation was carried out on ice for 1-2  
10    hours, instead of boiling water for 3-5 minutes, because the heating frequently caused the purified protein bands on the gel to smear. Substitution of LDS for sodium dodecyl sulfate resulted in greater resolution for the purified enzyme preparations. Discontinuous  
15    LDS/polyacrylamide gel electrophoresis (LDS-PAGE) was performed as described by Laemmli (1970) using 1.5-mm slab gels containing 8% acrylamide. The gels were stained with Coomassie brilliant blue or silver stained (Wray 1981). The molecular mass standards (Biorad) were phosphorylase b  
20    (97 Kd), bovine serum albumin (66 Kd), ovalbumin (42 Kd), carbonic anhydrase (31 Kd), soybean trypsin inhibitor (21 Kd) and hen egg white lysozyme (14 Kd).

Photographs of Coomassie blue or silver stained gels  
25    were digitized with an IBAS Interactive Image Analysis System (Zeiss, Thornwood, New York). The optical densities of the protein bands were measured by the image edit program.

30    Identification of Glycoproteins - Approximately 10  $\mu$ g protein of the EP<sub>1</sub> fraction purified from DS was separated by LDS-PAGE in an 8% gel. Subsequently, the periodic acid-Schiff base method using dansyl hydrazine (Eckhardt 1979) and FITC-labeled lectin method (Furlan 1979) were  
35    respectively used for glycoprotein identification.

-61-

Purification of the Cellulose Synthase - Gel filtration, anion exchange or Con A-Sepharose chromatography were separately combined with the cellulose entrapment step for the purification of the cellulose synthase. However, the recovery of enzyme activity was not high enough and the protein composition of the purified preparation was similar to that when directly purified by the cellulose entrapment (data not shown). Therefore, the cellulose entrapment method was used to purify the cellulose synthase.

The detergent-solubilized enzyme preparation was mixed with the substrate and the enzyme activator. After incubation at 30°C for 11 minutes, the reaction mixture was centrifuged, and most of the in vitro cellulose was sedimented. Enzyme activity was found primarily in the pellets. Generally, the ES<sub>1</sub> contained 20-30% of total protein in the pellets and showed only 10-15% of total enzyme activity. Over 80% of cellulose synthase activities still remained in the EP<sub>1</sub>. Therefore, the EP<sub>1</sub> and EP<sub>2</sub> were used as the samples of purified preparations for further characterization.

Cellulose synthase from Acetobacter is very labile. More than half of the activity of solubilized enzyme (such as DS, TS and OGS preparations) was lost after incubation, either at 4°C for 16 hours, or at 25°C for one hour. In contrast, entrapped enzyme (EP<sub>1</sub>) still maintains over half of its original activity after four hours at 25°C, while the activities in the ES<sub>1</sub>, DS and TS are almost completely lost. It is suggested that the entrapment of the enzyme by the in vitro cellulose product has a stabilizing effect.

As shown in Table III, the purification in the first entrapment step was 42-fold for EP<sub>1</sub>. The second

-62-

entrapment showed a 2 to 3-fold purification, in comparison with EP<sub>1</sub>. The total purification in both steps was about 96-fold. The recovery of cellulose synthase activity was low: 9% for the first entrapment, and 2% for the second entrapment. Therefore, each entrapment step lost 80% of enzyme activity. This may be indicative of

TABLE III

Purification of Cellulose Synthase from Acetobacter xylinum

Activity	Total Protein		Specific Activity		
	nmol/min/mg		nmol/min/mg		
Recovery Step	mg	%	Mean	Best	%
Washed membranes	396*	100	10		100
Triton extract (TSt)	65	16.4	36	45	59
Cellulose entrapment					
Step 1 (EP <sub>1</sub> )	0.85	0.2	420	630	9
Step 2 (EP <sub>2</sub> )	0.085	0.02	960	1560	2

\*Values were derived from 8 gm (dry weight) of cells.

#Mean specific activity represents the values from 5 sets of purifications. Best specific activity represents the value for the individual set of highest specific activity.

Gel Electrophoresis of the Purified Enzyme - The enzyme preparations from each purification step were analyzed by LDS-PAGE. In contrast to the protein bands from TS (Fig. 2, lane 1), very few of the protein bands over 66 Kd were observed from TSt (Fig. 2, lane 2). For the purified preparations from the trypsinized washed membranes, two major bands, 93 Kd and 83 Kd, were found in the gel from the EP<sub>1</sub> or EP<sub>2</sub> fraction (Fig. 2, lane 4 and 5). Overloading of EP<sub>2</sub> in the gel (Fig. 2, lane 5) produced 93 Kd and 83 Kd bands which accounted for 90% of the total density of stain in the gel lane. These two protein bands also were seen in the EP<sub>1</sub> fraction purified from TS, which

-63-

was prepared from the trypsin-untreated washed membranes (Fig. 2, lane 3).

5 In addition to two major protein bands, silver staining indicated some minor protein bands in the gel from the TSt-EP<sub>1</sub> fraction (Fig. 2, lane 6). For the most purified fraction, TSt-EP<sub>2</sub>, minor protein bands were still present in the gel (Fig. 2, lane 7 and 8). The 93 and 83 kd polypeptides also accounted for about 90% of the total stain density.

10 By using n-octyl glucoside or digitonin, and following a similar purification procedure, the EP<sub>1</sub> fractions also produced 93 Kd and 83 Kd bands as the major components (Fig. 3, lane 1, 2 and 3). Analysis of the protein composition in the EP<sub>1</sub> fraction from the DS with and without a cushion solution (Fig. 3, lane 3 and 4) revealed that more protein contaminants were removed in the presence of the cushion solution.

20 Without a reducing agent treatment, a 101 Kd band appeared instead of the 93 Kd band (Fig. 4, lane 1 and 2). Two small protein spots remained at the 93 Kd position (Fig. 4, lane 1, arrowheads). The 83 Kd band exhibited no change in mobility (Fig. 4, lane 1 and 2).

25 Trypsin Effect on the Enzymes - Incubation of either the washed membranes or TS with trypsin did not effect enzyme activity after 30 minutes at 25°C. Also, if the trypsin was added to the EP<sub>1</sub> fraction from DS, enzyme activity was still maintained after 1 hour at 25°C; however, only the 83 Kd polypeptide was still present in LDS-PAGE while the other polypeptides were degraded after 10 minutes incubation at 25°C (Fig. 4, lane 3, 4 and 5).

35

Characterization of the Glycosylated Protein - The binding of cellulose synthase to Con A-Sepharose was examined. The TS was applied and passed through the column. After washing with buffer, the cellulose synthase was bound with Con A. It could be easily eluted by addition of the enzyme buffer with 0.3 M methyl  $\alpha$ -D-mannopyranoside (Fig.

17% of the protein was recovered, and a 12-fold purification was achieved by this method.

When the purified EP<sub>1</sub> fraction from DS was separated by LDS-PAGE in the absence of reducing agents, and then stained using FITC-Con A, three bands of 110 Kd, 103 Kd and 83 Kd were found to react with the stain (Fig. 6, lane 2). The 83 Kd polypeptide fluoresced much more strongly than the other two bands. This band also fluoresced more strongly when stained with the Schiff-periodate method using dansyl hydrazine (Fig. 6, lane 1).

Gel Filtration Chromatography - The Triton-solubilized enzyme preparation, TS, was applied to a Sephacryl S-300 column for determination of the molecular weight of native cellulose synthase. The elution profile of cellulose synthase was determined by measuring the enzyme activity in the column fractions (Fig. 7A). There were two peaks of enzyme activity in the elution profiles. By the use of molecular weight markers as standards of Stokes' radii, the molecular weights of 490 Kd and 360 Kd for TS were shown to correspond to the two peaks of activity (Fig. 7A). The range was between 650 Kd to 300 Kd in TS. Therefore, the Stokes' radius of this enzyme was shown to be about 5.5-7.5 nm. After treatment with trypsin at 25°C for 10 minutes, the TS was applied to the column. The molecular weight range of the cellulose synthase was broadened to between 650 Kd and 100 Kd, and two peaks were

-65-

observed to correspond with the molecular weights of 490 Kd and 300 Kd (Fig. 7B).

After gel filtration, the recovery of cellulose activity was about 60% of total enzyme activity in the originally applied Triton-solubilized preparation (TS). For the trypsin-treated TS, there was no loss of enzyme activity as compared with total activity of the original sample. In addition, the specific activity of the peak fraction from trypsin treated sample was approximately twice as large as the nontreated sample (Fig. 7A and 7B).

**EXAMPLE II: IDENTIFICATION OF THE ACTIVE  
SUBUNIT OF CELLULOSE SYNTHASE**

Photoaffinity labeling - [ $\beta$ - $^{32}$ P]5N<sub>3</sub>UDP-glc was synthesized by a procedure described by Drake et al. (1989). In addition to 20  $\mu$ l of activator for cellulose synthase, each reaction mixture contained 50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, 1mM EDTA, the indicated protein quantity of enzyme sample, and 20  $\mu$ M of [ $\beta$ - $^{32}$ P]5N<sub>3</sub>UDP-glc (specific activity, 24 mCi/ $\mu$ mol) in a final reaction volume of 50  $\mu$ l at pH 8.0-8.5. For competition experiments, the competitor, namely UDP-glc, was added at final concentrations of 0, 50, 100, and 300  $\mu$ M, respectively. For saturation studies, [ $\beta$ - $^{32}$ P]5N<sub>3</sub>UDP-glc was added at final concentrations of 20, 50, 100, 300 and 500  $\mu$ M, respectively. Reactions were incubated for at 30 degrees centigrade for 30 seconds, followed by short wavelength UV irradiation (254 nm) with a hand-held UV lamp (Model UVSL-58, Ultraviolet Products, Inc.) in open Eppendorf microcentrifuge tubes at a distance of 4 cm for 60 sec. Reactions were terminated by the addition of 0.3 ml of 7% perchloric acid. After 20 minutes, the samples were centrifuged for 3 minutes in an Eppendorf model 5412 centrifuge. The pellet was resuspended in LDS sample

-66-

buffer for subsequent LDS-PAGE as previously described (Lin and Brown 1989). The Coomassie blue stained gel was dried and autoradiographed. Autoradiography was performed at -80°C with Kodak X-Omat AR film and a Dupont Cronex intensifying screen. The molecular mass standards (Bio-Rad) were phosphorylase b (97Kd), bovine serum albumin (66

The quantitation of the autoradiographs was measured by grey value discrimination of a digitized image (recorded on a Kontron/Zeiss IBAS Image Processing System). From this data, the corresponding molecular weight was calculated.

#### Results of Purification and Photoaffinity Labeling Studies

- Two crude enzyme preparations, membranous and digitonin-solubilized forms, and the purified enzyme were tested for their ability to photoincorporate [ $\beta$ -<sup>32</sup>P]5N<sub>3</sub>UDP-glc (Fig. 8). With or without the presence of c-di-GMP, there was no labeling of the 83 Kd band found in the membrane fraction except for the 57 Kd protein band (Fig. 8, lanes 1 and 2). A similar labeling of a 57 Kd band was observed in the digitonin-solubilized enzyme preparation (Fig. 8, lanes 3 to 4); however, a faintly photolabeled band corresponding to the 83 Kd polypeptide was seen in the presence of c-di-GMP (Fig. 8, lanes 2 and 4). In the purified preparation, the 83 Kd band became intensely labeled after azido UDP-Glu photolabeling (Fig. 8, lane 5) and the photoincorporation increased 45% more in the presence of c-di-GMP (Fig. 8, lane 6).

Figure 9 shows a Coomassie blue stained gel with the corresponding autoradiograph, demonstrating photolabeling of the cellulose synthase in various fractions purified using different detergents. In all cases, the 83 Kd protein band was exclusively photolabeled with [ $\beta$ -



-67-

5  $^{32}\text{P}$ ]5N<sub>3</sub>UDP-glc (Fig. 9B, lanes 2-4). Control experiments in which the reaction mixtures were not irradiated with UV light (Fig. 9A and 9B, lane 1) or in which pre-irradiated [ $\beta$ - $^{32}\text{P}$ ]5N<sub>3</sub>UDP-glc was used (Fig. 11, lane 1), yielded no labeling of the 83 Kd polypeptide or any other polypeptides.

10 UDP-glucose was known to be the glucosyl donor for cellulose synthase and was selected as a competitor against photoinsertion of [ $\beta$ - $^{32}\text{P}$ ]5N<sub>3</sub>UDP-glucose. As competitor concentrations increased from 50 to 300  $\mu\text{M}$  UDP-glc, the photoinsertion of the probe into the 83 Kd polypeptide became reduced (Fig. 10), with a Kd of 82  $\mu\text{M}$ . These UDP-glc protection studies provided further evidence to validate the specificity of the photoaffinity labeling.

20 A test of a probe's utility for active site studies has been shown to be the ability to saturate the available binding sites. Figure 11 shows the saturation of cellulose synthase photolabeling by [ $\beta$ - $^{32}\text{P}$ ]5N<sub>3</sub>UDP-glc, with a Kd value of 68  $\mu\text{M}$ . The photoinsertion of the probe into the 83 Kd protein band showed that saturation was being obtained with [ $\beta$ - $^{32}\text{P}$ ]5N<sub>3</sub>UDP-glc at concentrations just above 100  $\mu\text{M}$ . This demonstrated that the photoprobe was binding to a limited number of sites and that nonspecific photoinsertion under these conditions was minimal.

30 The 57 Kd band in the digitonin-extracted enzyme preparations was labeled either by using preirradiated [ $\beta$ - $^{32}\text{P}$ ]5N<sub>3</sub>UDP-glc solutions (Fig. 12, lane 1) or in the absence of photoactivation by UV irradiation (Fig. 12, lane 2), as well as by normal photolysis (Fig. 12, lane 3). These results indicated that the 57 Kd protein was not interacting with the photoprobe and that labeling was independent of a photoactive precursor. Furthermore, the labeling of this protein was inhibited in the presence of

500  $\mu$ M glc-1-P or glc-6-P (Fig. 12, lanes 4 and 5). It was not inhibited in the presence of glc-1,6-P (Fig. 12, lane 6) a fact consistent with the properties of phosphoglucomutase [Drake (1989); Ray and Peck (1972)].

5

There are three major requirements for demonstrating

studies: (a) only a specific protein(s) in an enzyme preparation is photolabeled with the photoprobe; (b) specific photoincorporation at the active site is measured by the ability of the probe to saturate the binding sites and by prevention of photolabeling with the native substrate at appropriate concentrations; and (c) the photolabeling is dependent on the presence of activating light to exclude pseudo-photoaffinity labeling.

The present inventor's results with the purified cellulose synthase preparation met all of these criteria. Thus, these studies confirmed the earlier suggestion (Lin and Brown 1989) and provided solid evidence that the 83 Kd polypeptide was the catalytic subunit of the cellulose synthase from Acetobacter xylinum strain ATCC 53582. Further strong evidence was that c-di-GMP substantially increased the photoincorporation of [ $\beta$ - $^{32}$ P]5N<sub>3</sub>UDP-glc with the 83 Kd protein. This cyclic nucleotide has been shown to be a true activator for cellulose synthase in A. xylinum (Ross 1987). The fibrillar structure and cellulosic nature of in vitro product has been visualized and characterized by electron microscopy (Lin 1985). Using X-ray diffraction analysis, the in vitro product from purified enzyme preparation or membrane fraction (Bureau and Brown 1987) was identified as cellulose II.

In the presence of the cellulose synthase activator, a weakly photolabeled 83 Kd polypeptide in the membrane fraction or the digitonin-solubilized preparation was

still detectable (Fig. 8, lanes 2 and 4). In contrast, no detectable labeling was observed when no activator was added (Fig. 8, lanes 1 and 3). Again, this demonstrated that c-di-GMP, the specific activator of cellulose  
5 synthase, truly increased the affinity of cellulose synthase for UDP-glc binding and produced a detectable photoactivated labeling of [ $^{32}\text{P}$ ]5N<sub>3</sub>UDP-glc. Two possible reasons for the weak photolabeling of the 83 Kd protein band from these enzyme preparations are as follows: (a)  
10 these crude preparations contain trace amounts of the cellulose synthase and therefore would be expected not to label as heavily as the purified enzyme preparation, and (b) the efficiency of photoinsertion as well as protein concentration affects this labeling.

15 The 57 Kd protein band labeled in the crude membrane fraction and the digitonin-solubilized enzyme preparation is close to the molecular weight previously reported for phosphoglucomutase in the Gram-negative bacteria (Ray and  
20 Peck 1972). Though phosphoglucomutase was reported as a soluble enzyme, labeling of a 62-64 Kd protein following incubation with [ $\beta$ - $^{32}\text{P}$ ]5N<sub>3</sub>UDP-glc has been observed in several membrane preparations (Drake and Haley, unpublished). This labeling has been attributed to trace  
25 levels of [ $^{32}\text{P}$ ]glc-1-P present in the photoprobe preparations or due to enzymatic hydrolysis of the photoprobe. Whether the 57 Kd protein was a membrane-bound isozyme of phosphoglucomutase or was an artifact of the membrane isolation technique was not determined.  
30 These experiments confirmed that the 57 Kd protein was a phosphoglucomutase.

**EXAMPLE III: USE OF THE AZIDO-UDP-GLUCOSE PHOTPROBES TO  
IDENTIFY GLUCAN SYNTHASES IN VASCULAR PLANTS**

Azido-UDP-Glc photolabeling of cotton fiber plasma  
5 membranes (+ UV but without activator/s) revealed only a  
weak photoinsertion of a 66 Kd polypeptide band, and  
occasionally, very faint bands. ~~These results prompted the~~  
~~inventors to attempt to enhance photolabelling~~  
as was successful in A. xylinum.

10 One approach which was attempted was similar to that  
attempted in A. xylinum. With activators from crude GX,  
an effector solution from Acetobacter which contains  
cyclic-di-GMP and other components, a significant increase  
15 was achieved in azido-UDP-Glc photoinsertion (Figure 14;  
lanes 3, 4, and 5). A 66 Kd band is prominently  
photolabeled, and two other bands, one at 57 Kd and the  
other at 35 Kd band are photolabeled.

20 In addition, a specific agent, a heated deproteinized  
supernatant derived from a membrane fraction of  
Acetobacter (called "crude factor"), fraction X-1 which  
has a maximum absorbance at 228 nm, is heat stable,  
ethanol soluble, and sensitive to phosphodiesterase (lane  
25 4). Azido-UDP-Glc photolabeling studies with the heated  
supernatant (lane 8) above showed an increase in  
photoinsertion of 5-fold for the 66 Kd band, 6-fold for  
the 57 Kd band, and 8-fold for the 35 Kd band. Pure  
cyclic-di-GMP, the specific allosteric activator for  
30 cellulose synthase in Acetobacter had no effect on azido-  
UDP-Glu photolabeling of the 66, 57, or 35 Kd bands (lane  
9).

35 Certain factors affect the intensity of the  
photolabeling of glucan synthase polypeptides. Azido-UDP-  
Glc photolabeling of these polypeptides is most intense in

-71-

preparations derived from fibers which are 30 days post anthesis. This period corresponds to the period of greatly increased secondary wall formation of virtually pure cellulose. Labeling of two of these proteins (35 Kd and 57 Kd) were abolished in dark controls (without UV irradiation, but with activator (lane 1)) and the 66 Kd band was weakly labeled under these conditions. Photolabeling of all three polypeptides was abolished in the presence of 1 mM unlabeled UDP-Glc, thus establishing specificity of azido-UDP-Glc photoaffinity binding (see Lin, et al, 1990 for comparable details).

Experiments with azido-UDP-Glc revealed that saturation of azido-UDP-Glc by photoinsertion was obtained at a concentration of approximately 100  $\mu$ M. The three photolabeled bands had differing apparent Kd's of 15  $\mu$ M for the 66 Kd band, 22  $\mu$ M for the 35 Kd band, and 25  $\mu$ M for the 57 Kd band.

All three polypeptides require either Ca++ or Mg++ to effectively photolabel. High Ca++ concentration coupled with low Mg++ or vice versa, do not significantly change azido-UDP-Glc photolabeling. However, when Mg++ is replaced by Mn++, photolabeling of the 35 Kd band is completely abolished while the 66 Kd band is greatly reduced. On the other hand, when Mg++ is replaced by Zn++, the photolabeling of the two bands does not change. The 66 Kd, 57 Kd, and 35 Kd bands are believed to represent at least two different glucan synthases.

**EXAMPLE IV: CLONING AND SEQUENCING OF THE GENE FOR THE CATALYTIC SUBUNIT OF CELLULOSE SYNTHASE FROM ACETOBACTER XYLINUM**

The purification of cellulose synthase activity and identification of the 83 Kd polypeptide by LDS-PAGE to be the catalytic subunit of cellulose synthase provided the

present inventors the necessary tools for attempting to clone the DNA encoding this important enzyme. N-terminal amino acid sequencing of the 83 Kd polypeptide was carried out as follows. Samples of the highly purified protein were loaded onto gels containing 8% polyacrylamide and electrophoresed according to Lin and Brown (1989). After

transferred to a polyvinylidene difluoride (PVDF) membrane (Matsudaria 1987). The 83-Kd polypeptide band was cut out with a clean razor. The protein from the excised bands were sequenced using an Applied Biosystems model 477A pulse liquid phase sequenator (carried out at the Department of Zoology at the University of Texas at Austin, Texas).

The amino acid sequence of the 83 Kd polypeptide was initially attempted on the purified cellulose synthase of the present invention without being treated with trypsin but this approach resulted in ambiguities at a number of positions during the sequencing and was not used in the design of the oligonucleotide probes. The initial sequence obtained is shown below: (Amino acid residues believed to be ambiguous are italicized.)

Met-Glu-Val-Arg-Gly/Ser-Leu/unknown-Thr-Gln-Leu-Arg-Leu-Gly-Asn-Thr-Leu-

While not of use for designing probes, the sequence above was useful in localizing the coding sequence eventually obtained.

The amino acid sequence of the 83 Kd polypeptide from purified cellulose synthase was obtained from trypsin-treated membrane preparations as described earlier herein. Specifically, trypsinized membranes were prepared as follows: to 10 ml of washed membranes (protein

-73-

concentration, 5 mg/ml), 0.2 mg of trypsin was added and gently shaken at 25°C for 30 minutes. After the addition of 0.2 mg of trypsin inhibitor for 20 minutes, the mixture was centrifuged at 18,000 xg for 20 minutes and washed  
5 once in TME.

The trypsinized membranes were resuspended in TME containing 20% glycerol, 0.6% (wt/vol) Triton X-100 to give a final protein concentration of 5.0 mg of membrane  
10 protein per ml. After homogenization with a teflon homogenizer, the suspension was gently shaken for one hour, followed by centrifugation at 100,000 xg for one hour. The supernatant represents the Triton-solubilized enzyme which is designated "TSt" since the trypsinized  
15 membranes are used.

The entrapment method used here was similar to that used previously (Lin and Brown 1989) and is described as follows: cellulose nitrate tubes, 9/16 x 3 3/4 in., were  
20 used. 2.6 ml of cushion solutions, consisting of TME buffer and 30% glycerol (v/v), were added to the bottom of the tubes. Subsequently, a mixture of 6.8 ml of the solubilized trypsin-treated enzyme, 0.2 ml of 100 mM UDP-glucose and 3.4 ml of N-Gx was layered on the top of  
25 cushion solution. The tubes were incubated in a 30°C water bath for 11 minutes then were placed on ice for two hours, and centrifuged for 20 minutes in a Beckman SW 40 Ti rotor at 50,000 xg. After overnight storage at -80°C, each pellet was resuspended in extraction buffer to 1/30  
30 the original loading volume and homogenized with a Teflon homogenizer. (Extraction buffer was prepared with 0.1% of detergent in TME buffer containing 20% glycerol). The suspension was centrifuged for 10 minutes at 15,000 xg. The supernatant was designated ES1. The EP1 was  
35 resuspended in extraction buffer to the same volume as ES1. The purified protein was then used for protein

-74-

sequencing studies as described above. The resulting trypsinized peptide sequence is shown below:

5                   Ser-Ser-Thr-Gln-Ser-Glu-Ser-Gly-Met-Ser-Gln-Trp/Leu-  
                  Met-Gly-Lys

10                   amino acid sequence of Fig. 1, the resulting sequence was  
                  fortuitously close to the amino terminal end of the  
                  cellulose synthase (amino acid residues 6-20). Once an  
                  amino acid sequence was determined with a sufficiently low  
                  level of ambiguity, design and synthesis of  
                  oligonucleotide (17-mers) from the amino acid sequence  
15                   data was undertaken for use as probes in DNA-DNA  
                  hybridization. For designing oligonucleotide probes from  
                  the amino acid sequence, the inventors selected a stretch  
                  of six amino acids (-Met-Ser-Gln-Trp/Leu-Met-Gly-) from  
                  the trypsin treated 83 Kd polypeptide sequence. Eight 17-  
20                   mer oligonucleotides, each with a unique sequence and  
                  having deoxyinosine (I) by the ambiguous position of the  
                  serine codons, were synthesized at Operon Technologies,  
                  Inc., California. The sequences of the oligonucleotide so  
                  constructed are given below:

25	83-1A	5'-ATGTCICAATGGATGGG-3'
	83-1G	5'-ATGTCICAGTGGATGGG-3'
	83-2A	5'-ATGAGICAATGGATGGG-3'
	83-2G	5'-ATGAGICAGTGGATGGG-3'
	83-3A	5'-ATGTCICAACTGATGGG-3'
30	83-3G	5'-ATGTCICAGCTGATGGG-3'
	83-4A	5'-ATGAGICAACTGATGGG-3'
	83-4G	5'-ATGAGICAGCTGATGGG-3'

35                   Hybridization of the oligonucleotide probes with A.  
                  xylinum DNA was carried out using the eight  
                  oligonucleotides that were thus synthesized. The probe



-75-

that showed maximum homology with DNA from A. xylinum ATCC 53582 was determined by DNA-DNA hybridization. In order to accomplish these studies, total DNA from A. xylinum ATCC 53582, A. xylinum ATCC 23769, and E. coli HB101 (a common strain of E. coli used in recombinant DNA experiments) was digested with HindIII and the fragments were separated on an agarose gel. After separation, the fragments were transferred by capillary action to a nylon membrane (GeneScreen; NEN Research Products) according to the procedure of Reed and Mann (1985). The DNA thus transferred was next hybridized with the <sup>32</sup>P-labeled oligonucleotide of the invention using standard procedures. Hybridization was accomplished in a solution with 5X SSC [1X SSC: 0.15M NaCl, 0.015M sodium citrate] at 45°C for 16-24 hours. The filters were then washed with a solution containing reduced salt concentration. In all cases, the hybridization temperature was 5-7°C below the dissociation temperature as described by Suggs, et al. (1981) applying the formula  $T_d = 2(A+T) + 4(G+C)$ .

Oligonucleotide 83-1G showed homology with a single HindIII fragment from A. xylinum strains ATCC 53582 and ATCC 23769 under stringent hybridization conditions during which none of the other oligonucleotides exhibited significant homology. To the surprise of the present inventors, all eight oligonucleotides showed hybridization to DNA from E. coli HB101 even under stringent conditions (at least 5 HindIII fragments hybridized). This fact prevented the use of standard procedures utilizing recombinant DNA libraries in E. coli.

That the 83-1G oligonucleotide was hybridizing with a unique DNA sequence from A. xylinum was confirmed by hybridizations with DNA digested with NruI, NarI, SmaI, ClaI, and BglII. In all cases, hybridization was observed with a single fragment of A. xylinum DNA.

-76-

Once the hybridization studies had been accomplished, cloning of the A. xylinum ATCC 53582 DNA fragment homologous to oligonucleotide 83-1G was carried out. The HindIII fragment of A. xylinum ATCC 53582 (~9.5 kb) showing homology to 83-1G oligonucleotide was cloned following isolation of A. xylinum DNA fragments in that

HindIII-cleaved pUC18. DNA fragments were isolated from low melting temperature agarose using the elution tip technique. The fragments were ligated with HindIII cleaved pUC18 using T4 DNA ligase (BRL) at 15°C for 8 hours. The ligation mixture was transformed into E. coli DH5-alpha where colonies carrying recombinant plasmids could be screened for the absence of  $\beta$ -galactosidase activity on medium with X-gal. Very few recombinants were obtained in this strain, possibly due to restriction of A. xylinum DNA in the Mcr+ background of this strain, and none of the recombinant plasmids carried the fragment homologous to 83-1G.

Therefore, the ligation mixture was next transformed into E. coli HB101 (a recA strain) and transformed colonies were selected on medium containing ampicillin (the selectable marker on pUC18). As pointed out earlier, this strain of E. coli made it difficult to screen colonies for recombinant plasmids since there was strong hybridization of the probes with the DNA of these cells. Therefore, plasmid DNA was isolated from pooled collections containing 50 Amp<sup>r</sup> colonies each and was analyzed by hybridization with <sup>32</sup>P-labelled 83-1G as probe. This screening resulted in the identification of the plasmid pIS532 that carried a HindIII fragment of 9.5 Kb from A. xylinum ATCC 53582 homologous to 83-1G.

Sequencing of the A. xylinum cellulose synthase catalytic subunit gene was accomplished by first

-77-

constructing a restriction map of pIS532 using various restriction enzymes. An EcoRI fragment of ~950 bp was identified by hybridization to carry the DNA sequence homologous to 83-1G oligonucleotide. This fragment was  
5 cloned into the vector M13mp19 in both orientations and DNA sequencing was performed by the dideoxy chain-termination method. The sequence of the 83-1G oligonucleotide was identified from the sequence obtained and the reading frame was established from the known amino  
10 acid sequence of the 83 Kd polypeptide. Cloning and sequencing of other fragments from pIS532 was done using M13mp18 and M13mp19 vectors. An analysis of the sequence obtained led to the identification of an open reading frame of 2169 bp coding for a polypeptide of approximately  
15 80 Kd. The first 20 amino acids deduced from the DNA sequence matched those obtained for the approximately 83 Kd polypeptide corresponding to the purified cellulose synthase subunit.

20 In summary, the DNA sequence for the catalytic-subunit of cellulose synthase from A. xylinum ATCC 53582 was identified using oligonucleotide probes designed from the amino acid sequence of an 83 Kd polypeptide that was obtained following purification of the cellulose synthase  
25 activity outlined in the examples above. A 9.5 kb HindIII fragment of A. xylinum DNA carrying the gene for the cellulose synthase catalytic-subunit was cloned in the vector pUC18. DNA sequencing was performed by the dideoxy chain-termination method following cloning of smaller  
30 fragments in M13mp18 and M13mp19. An open reading frame of 2169 bp coding for a polypeptide of 80 Kd was identified from the sequencing data in which the first 20 amino acids matched with the N-terminal amino acid  
sequence of the 83 Kd polypeptide.

35

-78-

**EXAMPLE V: CLONING AND SEQUENCING OF THE GENE  
ASSOCIATED WITH THE CATALYTIC SUBUNIT OF  
CELLULOSE SYNTHASE FROM ACETOBACTER XYLINUM**

5           *A. xylinum* strain ATCC 53582 was used for  
purification of the cellulose synthase and isolation of  
the 93 Kd polypeptide. [REDACTED]  
[REDACTED] gene for the 93 Kd polypeptide has been described above.  
N-terminal amino acid sequence of the 93 Kd polypeptide  
10           was determined in the same manner as described for the  
cellulose synthase catalytic subunit.

For DNA sequencing of the 93 Kd polypeptide coding  
region, fragments of pIS532 were cloned in M13mp 18 and  
15           M13mp19 in both orientations and deletion derivatives were  
prepared according to the method of Henikoff using the  
Erase-a-Base kit from Promega, Madison, WI. Sequencing  
was done by the dideoxy chain-termination procedure using  
the Sequenase version 2.0 kit from U.S. Biochemical Corp.,  
20           Cleveland, OH. DNA and amino acid sequences were analyzed  
using the PC/GENE programs and the Intelligenetics suite.

**Identification of the Gene for the 93 Kd Polypeptide  
From the N-Terminal Amino Acid Sequence**

25           Purification of cellulose synthase by the product  
entrapment procedure, using trypsin-treated membranes of  
*A. xylinum*, routinely are shown to possess two major  
polypeptide bands of MW 83 and 93 Kd. The N-terminal  
30           amino acid sequence of both these polypeptides was  
determined to help in the identification of these  
polypeptides and to design oligonucleotide probes for  
isolating the genes. The 83 Kd polypeptide was identified  
by photolabelling studies to be the catalytic subunit of  
35           cellulose synthase above, and the gene for this  
polypeptide was isolated using oligonucleotide probes.

-79-

DNA sequence beyond the stop codon of the cellulose synthase catalytic subunit gene, from plasmid pIS532, showed no transcription termination signal. Instead, an open reading frame with an ATG start codon was observed at position 2860, suggesting the presence of other gene(s) downstream of the catalytic subunit gene. Sequencing of the downstream region led to the identification of an open reading frame starting at position 2860 and terminating at position 5289, coding for a polypeptide of MW 86 Kd (Fig. 13). A potential ribosome-binding site with the sequence GGAACC is present 6 base pairs upstream of the putative start codon. The deduced amino acid sequence of this polypeptide from amino acid position 27 to 40 is found to be identical to the sequence of the first 14 N-terminal amino acids determined for the 93 Kd polypeptide that is shown here:

Ala-Pro-Ala-Trp/His-Gln-Pro-Glu-Ala-Thr/Ser-Asp-Leu-Pro-Pro-Leu-Arg/Leu.

The amino acid sequence identity between that determined for the 93 Kd polypeptide and one deduced from the DNA sequence confirms that this sequence codes for the 93 Kd polypeptide.

No N-terminal methionine is observed in the amino acid sequence determined for the 93 Kd polypeptide. The N-terminal sequence obtained could result from either cleavage of a signal peptide or from cleavage of a N-terminal fragment by trypsin used in the purification of the cellulose synthase. No N-terminal sequencing of the polypeptide which was purified from non-trypsin treated membranes was accomplished to determine if trypsin had cleaved an N-terminal fragment as was shown for the cellulose synthase catalytic subunit.

-80-

The amino acid sequence derived from the DNA sequence shows occurrence of a number of potential signal peptide cleavage sites. Potential cleavage sites at the N terminal end of the polypeptide are present at positions 14,15; 16,17; 18,19; 19,20; and 24,25. The cleavage site at position 16,17 showing the [REDACTED] (16,17) site and could be the actual cleavage site for the signal peptide.

The amino acid sequence at the N-terminal end of the processed polypeptide still differs from the amino acid sequence determined for the 93 Kd polypeptide, suggesting that a few more amino acids were removed following cleavage of the signal peptide. Since the 93 Kd polypeptide was purified from trypsin treated membranes, a short fragment of the processed polypeptide appears to be cleaved by trypsin at the N-terminal end. This is suggested by the amino acid sequence obtained for the 93 Kd polypeptide which is found to start after lysine at position 27 in the deduced amino acid sequence.

Even though the nucleotide sequences that are disclosed herein for the cellulose synthase catalytic subunit gene and the 93 Kd polypeptide gene are homologous to the sequences of the *bcsA* and *bcsB* genes respectively [Wong et al. 1990], they are not identical. This divergence could be due to the different strains of *A. xylinum* used in the two studies. Variability for cellulose production is commonly observed between different strains of *A. xylinum*, and this variability might very well be due to the differences in the DNA sequences coding for the various polypeptides involved in cellulose synthesis. Southern hybridization of DNA coding for the cellulose synthase catalytic subunit from *A. xylinum* ATCC 53582 with DNA from other strains of *A. xylinum* shows homology, but the size of DNA fragments

-81-

showing hybridization and the intensity of the hybridization signal is found to vary with the strains suggesting that there is a difference in the DNA sequences of these strains. Whereas some strains of A. xylinum seem  
5 closely related to one another, other strains are distantly related. Such variation of nucleotide sequences between different strains of bacteria is commonly observed, as in the case of the *trp* region in E. coli.

10 From the data disclosed herein, it was conclusively shown that the 83 Kd polypeptide, obtained from purified cellulose synthase and identified by photolabelling with [<sup>32</sup>P]5-azido UDP-glucose is the cellulose synthase  
15 catalytic subunit. Moreover antibodies raised against the 93 Kd polypeptide, when used in cellulose synthase enzyme assays, showed no effect on enzyme activity suggesting that the 93 Kd polypeptide was not the catalytic subunit.

20 Whether the cellulose synthase catalytic subunit can bind UDP-glucose on its own or requires some other polypeptides for the binding is not known at present. However, it is known that the activator c-di-GMP enhances the binding of UDP-glucose to the catalytic subunit  
25 suggesting that the activator-binding site is either present on the catalytic subunit or on another polypeptide that is tightly associated with it.

30 The present inventors propose that the cellulose synthase catalytic subunit and the 93 Kd polypeptide are tightly associated and are involved in the polymerization reaction. Other polypeptide(s), that probably are coded for by gene(s) in the cellulose synthesizing operon, may be loosely associated with the cellulose synthase complex  
35 and could therefore not be copurified with the cellulose synthase activity. These polypeptides may be involved in the crystallization process either directly by organizing

-82-

the glucan chains for efficient, directed export or indirectly by organizing the various polypeptides into a pore-like structure through which the glucan chain aggregates are guided facilitating the assembly of cellulose I microfibrils and ribbons.

**EXAMPLE VI: TRANSFORMATION OF COTTON WITH  
RECOMBINANT B-1,4 GLUCAN SYNTHASE**

The following example follows the method of Firoozabady (1987). It is proposed that this method may be used in combination with the present invention for transforming cotton with a recombinant glucan synthase gene and for obtaining transgenic plants therefrom. As a control for efficacy of the method when applied to glucan synthase, it is proposed that one may duplicate as closely as possible the method described below and make only those changes as are required from the inherent differences between the bacterial antibiotic resistance gene and the cellulose synthase gene.

It will be obvious to those of skill in the art that cotton and other plant species already possess functional glucan synthases. It is proposed here that certain utilities and advantages may be realized in improving the target host plant. This may be accomplished by introducing a modified (such as by site-directed mutagenesis or by construction of fusion proteins or by coupling with alternative promoter systems) homologous gene from the same plant species itself back into the same plant's cells. For instance, such a transformation might be accomplished by reintroducing the recombinant cellulose synthase gene derived from cotton by the methods and compositions of the present invention back into cotton cells but which gene was modified by placing the gene under the control of different promoters which then express the recombinant gene. Alternatively, the



-83-

transformations described here may be accomplished using recombinant cellulose synthase-encoding fragments from sources other than the host cell.

## 5     Bacterial Strains and Plasmids

Escherichia coli strains MC1061 (Casadaban and Cohen 1980) or K802 (Wood 1966) are employed in the recombinant DNA procedures. MM294(pRK2013) is employed as a plasmid-mobilizing strain (Ruvkun and Ausubel 1981) in triparental matings and 2174 (pPH1J1) as an excluding plasmid donor in marker exchange experiments (Garfinkel 1981). A. tumefaciens strains include 15955 (American Type Culture Collection) and LBA4404 (Hoekema 1983). E. coli strains are grown in L medium (Maniatis 1982) at 37°C and A. tumefaciens strains in YEP (1% yeast extract, 1% peptone, 0.5% NaCl) or Schilperoort's minimal (SM) medium (Klapwijk 1975) with 0.2% sucrose, at 28°C. Antibiotics (mg/l) are used for selecting E. coli and include: ampicillin, 50; kanamycin, 25; tetracycline, 10; gentamycin, 10. The latter three antibiotics are typically used for Agrobacterium selection at 25, 10, and 100mg/l, respectively and streptomycin at 250 mg/l. Plasmids pRK292 (Ditta 1985) and pTJS75 may be obtained from the laboratory of D. Helinski (University of California, San Diego).

## Binary Vector System

30       The details of the construction of the binary vector pH575 are available elsewhere (Sutton 1987). The cloning and recombinant DNA manipulations (Maniatis 1982) of pH575 and its derivatives are all performed using E. coli MC1061 as the host strain. Because this strain is resistant to streptomycin, it is unsuitable for use as a donor strain  
35       for conjugations into LBA4404. Consequently, the binary

-84-

vector is transformed into E. coli strain K802 for use in triparental matings (Kemp 1983).

5 For the control experiments, streptomycin- and kanamycin-resistant colonies are purified, and transconjugants are identified by ~~various~~

~~various~~ lysis procedure (Maniatis 1982).

10 The DNA between the border repeats of the transposon carrying the kanamycin gene is mobilized *in trans* for integration into plant cells by *vir* gene functions borne on the co-resident Ti plasmid pAL4404. For the purposes of integrating cellulose synthase into the plant cells, a suitable fragment of the cellulose synthase containing plasmid of Example III above (pIS532) is used to replace 15 the kanamycin gene in pH575 while maintaining the transposable characteristics of the end repeats. This plasmid is designated pH575-cellulose synthase.

#### Cis Vector System

20

As a control line of experiments, a *cis* vector system based on A. tumefaciens 15955 is constructed by using the T-region from pH575 to replace the wild-type Ti sequences of pTi15955. The 9530-bp HindIII-partial fragment of 25 pH575 that contains the T-region is re-cloned into the unique HindIII site of pRK292. The resulting plasmid, pH592, is then mated from K802 into a streptomycin-resistant spontaneous mutant of wild type 15955, and transconjugants 15955(pH592) are selected for resistance 30 to streptomycin, kanamycin, and tetracycline on SM medium. Single crossover events, by which a cointegrate plasmid containing pH592 and pTi15955 sequences is generated, is selected on SM by resistance to kanamycin, tetracycline and gentamycin following a second triparental mating in 35 which plasmid pPH1J1 is introduced into 15955(pH592). At least one of these colonies is then subjected to five

-85-

rounds of cycloserine enrichment as described (Koziel 1983), and at least approximately 2150 single colonies are screened for sensitivity to tetracycline. At least one colony is selected, and the expected structure of the T-DNA, resulting from a second crossover event that eliminates both the pRK292 sequences and T<sub>L</sub>-DNA bp 1016 to 11207 sequences (Barker 1983), is verified by DNA blot analysis.

10           A *cis* vector system based on *A. tumefaciens* 15955 is also constructed by using the T-region from pH575-cellulose synthase to replace the wild-type Ti sequences of pTi15955. The HindIII-partial fragment of pH575-cellulose synthase, or some other suitable fragment that  
15           contains the T-region is re-cloned into the unique HindIII site, or some other suitable site, of pRK292. The resulting plasmid is then mated from K802 into a streptomycin-resistant spontaneous mutant of wild type 15955, and transconjugants 15955(pH592-cellulose synthase)  
20           are selected for resistance to streptomycin and tetracycline on SM medium.

          Since kanamycin selection is not possible with the cellulose synthase marker, an approach similar to that  
25           used in the present invention including reprobing pooled plasmid DNA derived from transconjugants is used (Example III above). Single crossover events, by which a cointegrate plasmid containing pH592-cellulose synthase and pTi15955 sequences are generated, is selected on SM by  
30           resistance to tetracycline and gentamycin following a second triparental mating in which plasmid pPH1J1 is introduced into 15955(pH592-cellulose synthase). At least one of these colonies is then subjected to five rounds of cycloserine enrichment as described (Koziel 1983), and at  
35           least approximately 2150 single colonies are screened for sensitivity to tetracycline. At least one colony is

-86-

selected, and the expected structure of the T-DNA, resulting from a second crossover event that eliminates both the pRK292 sequences and T<sub>L</sub>-DNA bp 1016 to 11207 sequences (Barker 1983), is verified by DNA blot analysis.

5

#### Plant Material

Seeds of G. hirsutum cv. Coker 201 are surface-sterilized as described (Firoozabady and DeBoer 1986) except that seeds are exposed to bleach for only 8-10 minutes, germinated on MSO medium [hormone-free MS medium (Murashige and Skoog 1962), solidified with 0.2% Gel-rite (Kelco) and incubated as described (Firoozabady and DeBoer 1986).

15

#### Transformation and Selection

The bacteria used for inoculation of cotyledon segments are scraped off the agar medium and suspended to a concentration of  $\approx 10^8$  cells/ml in a cotton callus initiation liquid medium (G<sub>2</sub>) containing MS salts (Gibco), 100mg/l myo-inositol, 0.4mg/l thiamine HCl, 5 mg/l 6-( $\gamma$ , $\gamma$ -dimethylallylamino)-purine (2iP), 0.1 mg/l  $\alpha$ -naphthaleneacetic acid (NAA) (all from Sigma), 3% (w/v) glucose, pH 5.9. Cotyledon pieces ( $\approx 0.5$  cm<sup>2</sup> surface area) from sterile 12-day-old seedlings are dipped in the A. tumefaciens suspension in petri dishes and gently shaken for a few seconds to ensure contact of all cotyledon edges with the bacterial cultures. The cotyledon pieces are then blotted dry and placed on Whatman No. 1 filter paper on callus initiation medium G<sub>2</sub> solidified with 0.2% Gel-rite. Inclusion of the filter paper is not necessary for transformation but is suggested to greatly reduced bacterial overgrowth on plant tissues. Tissues are incubated at 25°C with a 16-h photoperiod (90  $\mu$ E m<sup>-2</sup> s<sup>-1</sup>).

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-87-

After three days cocultivation, cotyledon pieces are transferred to petri plates (without the filter paper) containing the same medium supplemented with 500 mg/I carbenicillin (to control bacterial growth) and, for control experiments, 15-35 mg/I kanamycin sulfate (U.S. Biochemicals). Tissues are incubated at 30°C with a 16-h photoperiod ( $10 \mu\text{E m}^{-2} \text{s}^{-1}$ ). After 3-4 weeks, calli are excised from the original explants, transferred to fresh medium, for control experiments to fresh kanamycin-containing medium, and incubated under lower light intensity ( $10 \mu\text{E m}^{-2} \text{s}^{-1}$ ).

#### Regeneration of Transgenic Plants

After two to three weeks, calli are placed and maintained on embryogenic medium ( $G_2$  with no hormones) and for experimental controls calli are placed under kanamycin selection (25 mg/I). Mature somatic embryos (5-10 mm in length with cotyledons, hypocotyl, and radicle structures) are transferred to GRMgn medium, which is a lower ionic strength medium (Stewart and Hsu 1977) modified by addition of 0.1 mg/l gibberellic acid (Sigma, filter-sterilized), 0.01 mg/l NAA (Sigma) and 0.5% glucose instead of sucrose (with no antibiotics), and incubated at 30°C, 16-h photoperiod ( $90 \mu\text{E m}^{-2} \text{s}^{-1}$ ) to germinate and form plantlets. Plantlets are transferred to GA-7 Magenta cubes (Magenta Corp.) containing GRMgn medium and incubated at 25°C with a 16-h photoperiod ( $90 \mu\text{E m}^{-2} \text{s}^{-1}$ ). Plants with 3 to 5 leaves are transferred to a commercial peat moss/perlite mixture (Growing Mix No. 2; Fafard Ltd., Canada), maintained in a growth chamber at 25°C night, 30°C day, 16-h photoperiod ( $150 \mu\text{E m}^{-2} \text{s}^{-1}$ ) and watered and fertilized as needed. Plants are gradually hardened off, repotted in soil and moved to a greenhouse.

-88-

TLC Octopine Assay

To detect octopine in callus or shoot material (as an indication of successful transformation), 10-20 mg fresh weight of tissue is incubated overnight on MSO medium

Tissues are homogenized in microfuge tubes, centrifuged (15000 g. 2 min), and 6  $\mu$ l of the supernatants is spotted (2  $\mu$ l at a time) on Whatman K-5 silica gel plates (20 x 20 cm) 1 cm apart. The plate is placed upright in a thin-layer chromatography (TLC) developing tank containing 20 ml of TLC buffer (methanol:2-butanol:0.1 M sodium acetate (pH 4.6), 15;1;4). After one hour, plates are removed, air-dried, sprayed faintly (under a fume hood) with a 1:1 fresh mixture of 10% NaOH in 60% ethanol: 0.04% phenanthrenequinone (Aldrich Chemical Co.) in 100% ethanol, and immediately visualized under UV light (254 nm).

The octopine assay described herein is suggested to be highly reliable. With this method, background artifacts or endogenous opines produced by feeding tissues on arginine (Christou 1986) are not anticipated to be present on the TLC plates at the time of scoring. This is due to the use of TLC buffer in which arginine migrates much more slowly on the plate than opines. It is also important that phenanthrenequinone staining be very light, and that the plate be read immediately after staining under UV light. The intensity of octopine production may be expected to vary, with some plants showing much stronger octopine production than others. In general, the improved sensitivity of the assay is likely to enhance the probability of detecting successful transformation of cotton callus with r combinant cellulose synthase.

**NPT II and Cellulose Synthase Assays: Enzyme-linked  
Immunoabsorbent assay (ELISA) and immunoblot**

5 NPT II (ncomycinphosphotransferase II, the enzyme  
which confers kanamycin resistance) is detected and  
quantified in cotton extracts by an ELISA assay. The  
ELISA is constructed with rabbit anti-NPT II  
immunoglobulin G (IgG), a biotin conjugate of this  
antibody (Guesdon 1986) and streptavidin alkaline  
10 phosphatase conjugate (Bethesda Research Laboratories).  
Rabbit anti-NPT II antiserum is raised against NPT II  
purified from E. coli containing plasmid pKS4 (Murai  
1983). Purified NPT II is used as the standard for  
calculating NPT II content in tissue extracts. Similarly,  
15 rabbit anti-cellulose synthase antiserum is raised against  
the highly purified cellulose synthase derived from A.  
xylinum or from the other cellulose synthase polypeptides  
of the present invention.

20 Plant extracts for ELISA is made by grinding four 5-  
mm leaf discs in 200  $\mu$ l of phosphate-buffered saline (127  
mM NaCl, 2.6 mM KCl, 8.5 mM  $\text{NaH}_2\text{PO}_4$ , 1.1 mM  $\text{KH}_2\text{PO}_4$ )  
containing 0.05% Tween-20, and 1% polyvinyl pyrrolidone  
40000 (Sigma). Extracts are centrifuged (15000 g, 5 min)  
25 and three three-fold dilutions of the sample supernatants  
are loaded into ELISA plates. Soluble protein  
concentrations are determined by the Bradford (Bradford  
1976) dye-binding assay (Biorad). NPT II levels are  
expressed as ng NPT II per mg of soluble protein in the  
30 extracts, while a similar measurement and analysis is  
carried out with cellulose synthase.

Cotton leaf extracts for immunoblot analysis are  
prepared in 4% SDS, 5% 2-mercaptoethanol, 20% glycerol in  
35 0.068 M Tris-HCl (pH 6.8), electrophoresed in 13%  
polyacrylamide gels (Laemmli 1970), and blotted to

-90-

nitrocellulose. Blots are processed in either rabbit anti-NPT II IgG (0.1  $\mu$ g/ml) or rabbit anti-cellulose synthase followed by goat anti-rabbit IgG alkaline phosphatase conjugate (0.2  $\mu$ g/ml, Kirkegarrrd and Perry) by standard procedures (Towbin 1979). Radioimmune

the wash buffer and RIPA containing 3% BSA and 1% goat serum is used as the antibody dilution buffer. Blots are developed in nitroblue tetrazolium/5-bromo-4-chloro-3-idolyl phosphate (both from Sigma) substrate solution (Balke 1984).

#### DNA Isolation and Blot Hybridization

DNA is prepared by isolation of nuclei from cotton leaf tissues using a modification of the method of Murray and Kennard (Murray and Kennard 1984). Young leaf tissues are ground in a Bellco glass-glass homogenizer at 4°C in nuclei buffer (20 mM 1,4-piperazinediethane sulfonic acid (pH 7), 3 mM  $MgCl_2$ , 0.5 M hexylene glycol, 10mM orthophenanthroline, 10 mM  $NaHSO_3$ ). Triton X-100 is added to a final concentration of 1% and the mixture centrifuged at 300 g, 4°C for 5 minutes. The nuclear pellet is resuspended in nuclei buffer and lysed by the addition of an equal volume of 30 mM EDTA, 1.5 M NaCl, and 1% cetyltrimethylammonium bromide in the presence of proteinase K (30  $\mu$ g/ml, Bethesda Research Laboratories) for 30 minutes at 65°C. DNA is precipitated in 2.5 M  $NH_4$ (Ac, 50% isopropanol precipitation. Five  $\mu$ g of DNA is digested with BamHI, electrophoresed through 0.8% agarose, and transferred (Southern 1975) onto nylon membrane (GeneScreen Plus, New England Nuclear). Hybridization is carried out with  $^{32}P$ -labeled synthetic OCS transcripts and nick-translated NPT II fragments from pH575, as a control, or with the various cellulose synthase probes of the invention. Filters are prehybridized overnight (in heat-



-91-

sealed bags) at room temperature in 100 mM  $\text{NaH}_2\text{PO}_4$  (pH 7.8), 20 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 5mM EDTA, 1mM orthophenanthroline, 0.1% SDS, 500  $\mu\text{g/ml}$  heparin sulfate, 10% sodium dextran sulfate, and 50  $\mu\text{g/ml}$  each, of heparin sulfate, herring sperm DNA and yeast RNA (Sigma). A portion of the  
5 prehybridization solution is removed from the bag, thoroughly mixed with the probe, and then returned to the bag containing the filter. The filter is hybridized for 6-12 h at 65°C and washed (20 MM  $\text{NaH}_2\text{PO}_4$ , 5 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 1 mM  
10 EDTA and 0.1% SDS) at 65°C for 1 h with five buffer changes. Autoradiography is performed on Kodak XAR-5 film with an intensifier screen at -70°C.

Plants whose tissue extracts indicate the presence of  
15 cellulose as measured above are selected and tested for enhanced cellulose fiber production. Where applicable, those plants demonstrating improved characteristics will be crossed in order to produce transgenic seed. Any number of indicia may be important to improvement of the  
20 plant species thus transformed.

Enhanced or altered cellulose production as a result of the recombinant DNA experiments is deduced by any one or any combination of any of several tests in which the  
25 control (non-transformed) and experimental (transformed) plants are compared:

(a) The amount of total cellulose per ovule or per fiber is compared, on a dry weight basis, either  
30 before or after alkaline extraction to remove non-cellulosic substances from the cell wall;

(b) The amount of cellulose per fiber as based on a ster ology analysis (Zeiss IBAS Image Analysis and  
35 AImage Processing System and Image Analysis manual)

-92-

of cotton fiber cell wall thickness and fiber length or cotton fiber volume is compared;

(c) The polymorph of cellulose present is compared, as determined by x-ray diffraction of untreated

cellulose (see procedures described by Alfred D. French in "Cellulose Chemistry and its Applications", Ed. T.P. Nevell and S.H. Zeronian (1985), John Wiley and Sons, New York, Chapter 3, pp 84-111); Also, "Electron Diffraction: An Introduction for Biologists", by D.L. Misell and E.B. Brown, Vol. 12, Practical Methods In Electron Microscopy, Ed. A.M. Glauert (1987), Elsevier, New York, p. 287). Such polymorphs in addition to the native cellulose I might include but may not be limited to cellulose II, cellulose III or cellulose IV (note: only cellulose I is expected to be synthesized in vivo in the control plants);

(d) Changes in the average molecular weight of the cellulose are compared, using procedures known to those of skill in the art such as those described by S.B. Ross-Murphy in "Cellulose Chemistry and Its Application", Ed. T. P. Nevell and S.H. Zeronian (1985), John Wiley and Sons, New York, Chapter 8, pp. 202-222;

(e) X-ray or electron diffraction patterns of cellulose are compared as described in (c) above to determine changes in the percent crystallinity of the cellulose;

(f) Changes in total plant size or changes in specific organ size such as cotton boll size, ovule siz , leaf size, stem size, root size, and the like

-93-

as reflected by average cell size within the various tissues and organs of the plant are compared;

5 (g) Ultrastructural morphology of the cellulose, either in the form of microfibrils, elementary fibrils, sub-fibrils, and the like, are compared using transmission electron microscopy and the typical processing known as "negative staining" or standard heavy metal replication techniques whereby the cellulose is made visible for analysis and measurement are carried out [Typical procedures are those found in *Experimental High-Resolution Electron Microscopy* (second edition) by John C.H. Spence, p. 427, Oxford University Press, New York, 1988];

15 (h) Ultrastructural morphology of the cellulose synthesizing complexes is compared, using the freeze-etch/freeze fracture method, as exemplified in "Replica, Shadowing and Freeze-Etching Techniques" by J.H.M. Willison and A.J. Rowe, Vol. 8 of *Practical Methods In Electron Microscopy* Ed. A.M. Glauret, North-Holland Publishing Co., New York (1980) p. 301. See also, "Cellulose Biogenesis and a Decade of Progress: A Personal Perspective", by R. Malcolm Brown, Jr. (1989) pp. 639-657, In *Cellulose and Wood-Chemistry and Technology*, Ed. C. Schuerch, John Wiley and Sons, New York, p. 1638;

30 (i) Uptake of cellulose direct dyes and/or fluorescent brightening agents is compared in which case, the patterns of the controls and experimental plants are differentiated, suggesting changes or alterations in cellulose crystallinity, orientation, etc. See Haigler et al. (1980) "Calcofluor White ST Alters the In vivo Assembly of C llulose Microfibrils", *Science*, 210:903-906; and

-94-

(j)  $^{13}\text{C}$ , carbon-solid state NMR analysis is carried out to differentiate  $I_{\alpha}$  and  $I_{\beta}$  forms of the native cellulose polymorph [Atalla (1984), pp. 881-891, *Structure, Friction and Biosynthesis of Plant Cell Walls*, Ed. Dugger and Bartnicki-Garcia].

The references noted in (a) - (j) above, to the extent that they provide procedural details supplementary to those set forth herein, are specifically incorporated herein by reference.

**EXAMPLE VII: PROBING PLANT DNA FOR B-1,4 GLUCAN SYNTHASE HYBRIDIZING TO RECOMBINANT B-1,4 GLUCAN SYNTHASE PROBES**

The following example is patterned after the method of Dellaporta et al. (1985) and is proposed as general method for isolating and probing plant DNA with which the methods and compositions of the present invention may be combined. It is suggested to be used herein for probing plant DNA for glucan synthase hybridizing to recombinant glucan synthase probes such as the cellulose synthase or cellulose synthase-related protein described above.

In general, this protocol is a rapid microscale method for isolation of plant DNA without the use of ultracentrifugation of CsCl gradients. The DNA produced is of moderately high molecular weight and serves as a satisfactory substrate for most restriction endonucleases and is suitable for genomic blot analysis. In addition to the rapidity and convenience of minipreps which permit a large number of samples to be processed in just a few hours, the small amount of tissue required (less than 1.0 gram) allows for molecular analysis of plants at a very young stage. Miniprep DNA yields from leaf tissue of most species tested in the prior art are typically 40-100 ug

-95-

per gram of tissue, greater than 50 kb in average length, and remarkably uniform from sample to sample.

Several modifications have been applied to the method described herein in order to extend the application of miniprep procedures to other plant species and it is anticipated that one may use similar alterations in order to fit the technique to specific species. The selection of a particular protocol depends to a large degree on the plant species used. However, the procedure reported here is selected to be suitable for most situations.

#### Procedure

Approximately 1 gm of leaf tissue is quick frozen in liquid nitrogen and ground to a fine powder in a 3" mortar and pestle. The resulting powder is transferred with liquid nitrogen into a 30 ml Oak Ridge tube. It is imperative not to let the tissue thaw once frozen until the buffer is added and not to cap the tubes while nitrogen is evaporating.

Next, 15 ml of Extraction Buffer (EB: 100 mM Tris pH 8, 50 mM EDTA pH 8, 500 mM NaCl, 10 mM mercaptoethanol) is added. For maximum DNA yields, the cells are further broken by grinding the mixture at a low setting (about 3) with a Polytron (Brinkmann Instruments, Inc.). This step is optional and is tested for its efficacy prior to routine use on a specific tissue source.

Approximately 1.0 ml of 20% SDS is added to the tubes and is mixed thoroughly by vigorous shaking. The tubes are then incubated at 65°C for 10 minutes. A volume of 5.0 ml of 5 M potassium acetate is then added to the tubes and the tubes are shaken vigorously and incubated 0°C for 20 minutes.

-96-

This procedure is expected to remove most proteins and polysaccharides as a complex with the insoluble potassium dodecyl sulfate precipitate. Precipitation from 0.3 M sodium acetate using relatively small amounts of isopropanol (about 0.6 volumes) has been reported to

(Marmur, 1961). The sodium acetate also yields a tight fibrous precipitate that is easily washed and dried. The DNA dissolves readily if allowed to rehydrate at 4°C for 1 hour followed by light vortexing.

The tubes are then centrifuged at 25,000 x g for 20 minutes. The supernatant is poured through a Miracloth filter (Calbiochem) into a clean 30 ml tube containing 10 ml isopropanol. The resulting solution is mixed and incubated at -20°C for 30 minutes.

The DNA is pelleted at 20,000 x g for 15 minutes. The supernatant is gently decanted and the resulting pellets are lightly dried by inverting the tubes on paper towels for 10 minutes. DNA pellets are redissolved with 0.7 ml of 50 mM Tris, 10 mM EDTA, pH 8 and the solution is transferred to an Eppendorf tube. These tubes are then centrifuged in a microfuge for 10 minutes to remove insoluble debris.

The supernatant is then transferred to a new Eppendorf tube containing 75 µl 3M sodium acetate and 500 µl isopropanol, mixed well and the clot of DNA is pelleted in a microfuge for 30 seconds. The pellet is then washed with 80% ethanol, dried, and redissolved in 100 µl of 10 mM Tris, 1 mM EDTA, pH 8.

Minipreps can be stored for several months without evidence of degradation and can be restricted with a variety of restriction enzymes and ligated without further

-97-

purification. It has been found previously that 10.0  $\mu$ l of miniprep DNA is sufficient for a single 8 mm lane in an agarose gel which is to be used for filter hybridization with single-copy probes. Heat-treated RNAase must be added to the restriction reaction to digest contaminating RNA in each prep. Hence, a typical reaction contains the following:

	Miniprep DNA	10.0 $\mu$ l
10	10X Restriction Buffer	3.0 $\mu$ l
	0.5 mg/ml RNAase	2.0 $\mu$ l
	<u>EcoRI</u>	8 units
	dH2O to 30 $\mu$ l.	

15 Digestion is usually complete after 3 hours at 37°C. Occasionally, minipreps are difficult to digest with certain enzymes. This problem can be overcome by adding 5.0  $\mu$ l of 0.1 M spermidine to the entire miniprep before digestion (Focus 1982).

20 \*\*\*\*\*

Studies by the present inventors and others support the conclusion that many cellulose synthases will be similar enough in primary structure to allow detection by the technique outlined above or by similar techniques. For instance, based on the similarities observed between the terminal synthesizing complexes found at the growing tip of microfibrils of cellulose and similarities in the cellulose organization, it has been possible to construct a phylogenetic pathway for cellulose biogenesis. Where such similarities are adequate, the methods and compositions of the present invention allow the detection and isolation of the similar gene and/or the similar polypeptide.

**EXAMPLE VIII: CLONING OF  $\beta$ -1,4 GLUCAN SYNTHASE GENES  
IN CYANOBACTERIAL/E. COLI SHUTTLE VECTORS**

Using the methods and compositions of the present invention, two recombinant plasmids are constructed for transfer of a  $\beta$ -1,4 glucan synthase such as the ~~cellulose synthase gene from *A. xylinum*~~ ~~cellulose synthase gene from *A. xylinum*~~. First, a DNA fragment from the plasmid pIS532, carrying the intact cellulose synthase gene along with the promoter sequences from *A. xylinum*, is cloned in the shuttle vector pUC303 (Kuhlemeier et al. 1983). Expression of the cellulose synthase gene is under the control of the *A. xylinum* promoter in this recombinant plasmid. Second, the coding region of cellulose synthase is cloned in the shuttle vector pPL191 (Friedberg 1988) where it is under the control of the operator promoter regions  $O_L P_L$ ,  $O_R P_R$ , and the temperature-sensitive repressor gene cI857 of bacteriophage lambda. These regulatory sequences are used for monitoring activity of reporter genes in a cyanobacterium background (Friedberg and Seijffers, 1986).

**Transformation of *Anacystis Nidulans* R2-SPc**

*Anacystis nidulans* R2 is the strain of choice for molecular genetic studies in cyanobacteria for which a complete system of gene cloning and analysis has been developed. This strain carries two plasmids of molecular weights 8 Kb and 50 Kb. The 8 Kb plasmid (pUH24) is used for the construction of most shuttle vectors, including pUC303 and pPL191 mentioned above. In order to avoid recombination between the vector and the pUH24 present in *A. nidulans* R2, use is made of the pUH24-cured strain, R2-SPc, for transformation with these vectors.

*A. nidulans* R2-SPc is grown in BG-11 medium (Rippka et al. 1979) at 30°C with continuous illumination of 250-



-99-

800 foot candles of fluorescent or mixed tungsten-fluorescent light. Cells from a 30 ml culture are pelleted by centrifugation at 5000 rpm in a Sorvall SS34 rotor for 5 minutes. The pellet is resuspended in 15 ml of 10mM NaCl and the centrifugation repeated. The pellet is finally resuspended in 3 ml of BG-11 medium ( $5 \times 10^8$  to  $1 \times 10^9$  cells/ml). 300  $\mu$ l aliquot of cells are transferred to sterile 1.5 ml Eppendorf tubes and 10 ng to 1  $\mu$ g of recombinant plasmid DNA is added. The tubes are then incubated in the dark at 30°C for 4-16 hours with gentle agitation, following which 100-150  $\mu$ l aliquots of each transformation reaction are spread on 100 mm plates containing 40 ml of BG-11 agar. The plates are incubated under standard illuminated growth conditions for 4-6 hours prior to the addition of the required selective agent. For pUC303 derived plasmid, either chloramphenicol (7.5  $\mu$ l/ml) or streptomycin (10  $\mu$ g/ml) is used. Where transformation has been done with pPL191 derived plasmid, kanamycin (25  $\mu$ g/ml) is used for selecting the transformed cells. The antibiotics are added by lifting the agar slab with an alcohol-flamed spatula and dispensing 400  $\mu$ l of a 100x concentrated stock underneath. Transformed colonies typically appear within 4-7 days of incubation in the light at 30°. The transformants are purified on BG-11 agar plates containing the suitable antibiotic, and analyzed for their plasmid content.

#### **Expression of A. Xylinum Cellulose Synthase Gene in A. Nidulans**

30

The expression of cellulose synthase gene from its own promoter (cloned in pUC303) and the bacteriophage lambda promoter (cloned in pPL191) are monitored at the level of RNA synthesis, cellulose synthase polypeptide synthesis, cellulose synthase activity and cellulose biosynthesis. RNA from the transformed cells is isolated

35

-100-

essentially as described by Golden et al. (1987) and analyzed by Northern analysis. The synthesis of the cellulose synthase polypeptide in these transformants is determined by western blot analysis using polyclonal

5 antibodies raised against the A. xylinum cellulose synthase. Cellulose

was used as the substrate according to standard procedures herein. Whether the transformed cells synthesize cellulose is determined by isolating the alkali-insoluble

10 material from these cells and analyzing it by X-ray diffraction.

**EXAMPLE IX: CELLULOSE SYNTHASE ENZYME COMPLEXES  
PRODUCE CELLULOSE I PRODUCT**

15

Standard conditions for isolation and preparation of membrane fractions from Acetobacter were used (see Lin and Brown, 1989). Membrane fractions from disintegrated cells (using the French Press) were treated with trypsin as

20 listed in the protocol of Lin and Brown, 1989. This was called the "MF" fraction. Unlike Lin and Brown, 1989, the MF fraction was treated with 0.6% Triton to solubilize the membrane proteins. To this mix were added the ingredients for the in vitro reaction mixture which included Ca++,

25 Mg++, UDP-glc, and purified cellulose synthase activator, cyclic di-GMP. Unlike the usual procedures, no membranes were separated by differential centrifugation, and the entire reaction mixture with membranes was allowed to synthesize cellulose in vitro.

30

The cellulose product (as shown in Fig. 15) is in the form of extended subfibrils with uniform dimensions of approximately 2.0 x 2.0 nm x and at least 200nm in length. This provides clear proof that the in vitro synthesized

35 cellulose polymorph is cellulose I, the same type and morphology of that which is assembled in vivo. Cellulose

-101-

I polymorphs can only be synthesized in vitro when the UDP-glc substrate concentration is sufficiently high (in this case, 20.0mM). If this substrate is used in a lower concentration range such as 1.9mM, only cellulose II is  
5 synthesized as shown in Lin and Brown, 1989 (also see Bureau and Brown 1987). The rodlets of cellulose II are only 35 nm long and have variable widths, up to 5.4nm.

By sufficiently saturating all of the available UDP-  
10 glc binding sites of the isolated enzyme complex, multiple parallel glucan chains are polymerized in sufficient close proximity to crystallize into the metastable cellulose I polymorph. If these sites are undersaturated, the glucan chains may have the capacity to fold and collapse into the  
15 more stable cellulose II polymorph. Cellulose I can be converted to cellulose II (and not vice versa) by the process of mercerization or treatment with concentrated alkali which dissolves the crystalline cellulose, then as the alkali is removed, precipitates to form the more  
20 stable cellulose II polymorph.

It should be noted that the 2.0 x 2.0nm subfibril is itself too small to be highly crystalline; however, if multiple subfibrils coalesce as they do in vivo, a stable,  
25 highly crystalline cellulose I polymorph is formed. Thus, a necessary prerequisite for cellulose I formation in vivo or in vitro is the formation of a stable, extended subfibril of relatively constant dimensions, and the enzyme complex must be fully saturated with substrate to effect  
30 this synthesis.

The present invention has been described in terms of particular embodiments found or proposed by the present inventors to comprise preferred modes for the practice of  
35 the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure,

-102-

numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. For example, due to codon redundancy, changes can be made in the underlying DNA sequence without affecting the protein sequence.

Moreover, due to biological considerations, changes can be made in the protein structure without affecting in kind or amount of the biological action. All such modifications are intended to be included within the scope of the appended claims.

\* \* \* \* \*

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-109-

## CLAIMS:

1. A recombinant vector incorporating a DNA segment having a sequence encoding a  $\beta$ -1,4 glucan synthase polypeptide.

2. The vector of claim 1, where the sequence encodes a polypeptide which includes the amino acids 1 to 723 of Figure 1.

3. The vector of claim 2 where the sequence encodes a polypeptide consisting essentially of amino acids 1 to 723 of Figure 1.

4. The vector of claim 1, where the sequence encodes a polypeptide which includes the amino acids 1 to 809 of Figure 13.

5. The vector of claim 4 where the sequence encodes a polypeptide consisting essentially of amino acids 1 to 809 of Figure 13.

6. The vector of claim 1, where the  $\beta$ -1,4 glucan synthase polypeptide encoding sequence is positioned adjacent to and under the control of an effective promoter.

7. The vector of claim 6, where the promoter comprises a prokaryotic promoter, the vector being adapted for expression in a prokaryotic host.

-110-

8. The vector of claim 6, where the promoter comprises a eukaryotic promoter, the vector being adapted for expression in an eukaryotic host, and the vector further includes a polyadenylation signal positioned 3' of the carboxy-terminal amino acid, and within a transcriptional unit of the gene.

9. A DNA segment having a sequence encoding a  $\beta$ -1,4 glucan synthase polypeptide.

10. The DNA segment of claim 9, where the sequence encodes a polypeptide corresponding to amino acids 1 to 723 of Figure 1.

11. The DNA segment of claim 9 where the sequence encodes a polypeptide consisting essentially of amino acids 1 to 723 of Figure 1.

12. The DNA segment of claim 9, where the sequence encodes a polypeptide corresponding to amino acids 1 to 809 of Figure 13.

13. The DNA segment of claim 9, where the sequence encodes a polypeptide consisting essentially of amino acids 1 to 809 of Figure 13.

14. A DNA segment having a sequence comprising at least a 14 base sequence of the DNA sequence of Figure 1, or its complement, the segment capable of hybridizing to the DNA sequence of Figure 1, or its complement.

-111-

15. The DNA segment of claim 14, where the sequence comprises at least an 18 base sequence of the DNA sequence of Figure 1.

5

16. A DNA segment having a sequence consisting essentially of at least a 14 base sequence of the DNA sequence of Figure 1.

10

17. A DNA segment having a sequence comprising at least a 14 base sequence of the DNA sequence of Figure 13, or its complement, the segment capable of hybridizing to the DNA sequence of Figure 13, or its complement.

15

18. The DNA segment of claim 17, where the sequence comprises at least an 18 base sequence of the DNA sequence of Figure 13.

20

19. A DNA segment having a sequence consisting essentially of at least a 14 base sequence of the DNA sequence of Figure 13.

25

20. A recombinant vector incorporating a DNA segment as defined by any one of claims 9 through 19.

30

21. A  $\beta$ -1,4 glucan synthase polypeptide.

-112-

22. The  $\beta$ -1,4 glucan synthase polypeptide of claim 21 further defined as including amino acids 1 to 723 of Figure 1.

5

23. The  $\beta$ -1,4 glucan synthase polypeptide of claim 21 further defined as a recombinant polypeptide.

10 24. The  $\beta$ -1,4 glucan synthase polypeptide of claim 21 further defined as including amino acids 1 to 809 of Figure 13.

15 25. The  $\beta$ -1,4 glucan synthase polypeptide of claim 21 further defined as a recombinant polypeptide.

20 26. An antibody immunologically reactive with any one of the polypeptides of claim 21 through 24.

25 27. The antibody of claim 25, further defined as a monoclonal antibody.

28. A method of purifying a  $\beta$ -1,4 glucan synthase polypeptide, comprising:

- 30 (a) culturing cells capable of producing  $\beta$ -1,4 glucan polymers so that  $\beta$ -1,4 glucan synthase is expressed;
- 35 (b) partially purifying the  $\beta$ -1,4 glucan synthase from the cells by solubilizing the cellular membranes;

-113-

(c) identifying an active subunit of the  $\beta$ -1,4 glucan synthase; and

5 (d) further purifying the active subunit of the  $\beta$ -1,4 glucan synthase.

10 29. The method of claim 28 where the cells capable of  $\beta$ -1,4 glucan polymer synthesis are cells of cotton.

15 30. The method of claim 28 where further purifying the active subunit of the  $\beta$ -1,4 glucan synthase comprises purifying the  $\beta$ -1,4 glucan synthase by product entrapment.

20 31. The method of claim 28 where identifying the active subunit of  $\beta$ -1,4 glucan synthase further comprises admixing a  $\beta$ -1,4 glucan synthase activator with the partially purified  $\beta$ -1,4 glucan synthase.

25 32. The method of claim 28 where identifying the active subunit of  $\beta$ -1,4 glucan synthase further comprises photoincorporating onto the partially purified  $\beta$ -1,4 glucan synthase a radioactively labelled azidonucleotide analog capable of specifically interacting with the nucleotide binding site of  $\beta$ -1,4 glucan synthase.

30 33. The method of claim 28 where further purifying the active subunit of the  $\beta$ -1,4 glucan synthase comprises purifying the  $\beta$ -1,4 glucan synthase by electrophoresing the labeled  $\beta$ -1,4 glucan synthase through a gel matrix.

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-114-

34. The method of claim 28 where the cells capable of  $\beta$ -1,4 glucan polymer synthesis are cells of a strain of Acetobacter xylinum, cells of an Agrobacterium species, cells of a Rhizobium species or cells of a Sarcina species.

35. The method of claim 28 where partially purifying  $\beta$ -1,4 glucan cellulose synthase from the cells by solubilizing the cellular membranes is accomplished using digitonin.

36. The method of claim 28 where the  $\beta$ -1,4 glucan synthase activator is bis-(3',5')-cyclic diguanylic.

37. The method of claim 28 where the  $\beta$ -1,4 glucan synthase activator is c-di-GMP.

38. The method of claim 28 where the  $\beta$ -1,4 glucan synthase activator is contained in a crude extract of A. xylinum cells.

39. A recombinant host cell which incorporates a DNA sequence in accordance with claim 9.

40. A recombinant host cell which incorporates DNA sequences encoding more than one glucan synthase polypeptide.

41. The recombinant host cell of claim 39 further defined as a eukaryotic host cell.



-115-

42. The recombinant host cell of claim 39 further defined as a prokaryotic host cell.

5

43. The prokaryotic host cell of claim 42 further defined as a cyanobacterium.

10

44. The recombinant host cells of claims 39 through 43 where the DNA segment encoding a  $\beta$ -1,4 glucan synthase polypeptide is under the transcriptional control of regulatory signals functional in the recombinant host cell which regulatory signals appropriately control the expression of the glucan synthase polypeptide in a manner to allow all necessary transcriptional and post-transcriptional modification.

15

20

45. The recombinant host cell of claim 40 where the host cell is a plant entity consisting essentially of a plant cell, seed, or plant having a DNA segment, as a result of the in vitro introduction of the DNA segment into a plant cell, the DNA segment encoding a  $\beta$ -1,4 glucan synthase polypeptide, the DNA segment being under the transcriptional control of regulatory signals functional in the plant entity, which regulatory signals appropriately control the expression of the  $\beta$ -1,4 glucan synthase polypeptide in a manner to allow all necessary transcriptional and post-transcriptional modification.

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30

46. The recombinant plant cell, seed or plant of claim 43 which comprises a specie of the genus Gossypium.

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-116-

47. The recombinant plant cell, seed or plant of claim 43 which comprises an algae.

5           48. The gene of claim 43 which comprises a gene which is heterologous to the gene of claim 43. The gene is introduced.

10           49. A method of producing a  $\beta$ -1,4 glucan synthase polypeptide, comprising:

15                   (a) producing a recombinant host cell according to any of claims 38 through 46, such cell being capable of expressing the polypeptide;

20                   (b) culturing the host cell under conditions appropriate for expressing the polypeptide; and

                 (c) recovering the polypeptide.

25           50. A method of producing a  $\beta$ -1,4 glucan polymer, comprising:

30                   (a) producing a recombinant host cell according to any of claims 38 through 46, such cell being capable of producing the  $\beta$ -1,4 glucan polymer;

35                   (b) culturing the host cell under conditions appropriate for producing the  $\beta$ -1,4 glucan polymer; and

-117-

(c) recovering the  $\beta$ -1,4 glucan polymer.

51. The method of claim 50 where the  $\beta$ -1,4 glucan  
5 polymer is further defined as cellulose.

52. The method of claim 50 where the  $\beta$ -1,4 glucan  
polymer is a mixed  $\beta$ -1,4 glucan polymer.  
10

53. A method of producing a  $\beta$ -1,4 glucan polymer,  
said polymer further comprising a predetermined amount of  
a given  $\beta$ -1,4 glucan polymorph, comprising:  
15

- (a) selecting a cell capable of producing  $\beta$ -1,4  
glucan polymer;
- (b) culturing the cell under conditions  
20 appropriate for producing the  $\beta$ -1,4 glucan  
polymer;
- (c) obtaining a cell-free extract of the cell;
- (d) providing a at a selected concentration  
25 substrate to the cell extract designed to  
cause the cell extract to form the selected  
 $\beta$ -1,4 glucan polymorph; and
- (e) recovering the selected  $\beta$ -1,4 glucan  
30 polymer.

54. The method of claim 53 where the polymorph is  
35 cellulose I.

-118-

55. The method of claim 53 where the substrate is selected from the group of nucleotide sugars consisting of UDP-xylose, UDP-mannose, ADP-glucose, GDP-glucose, and UDP-glucose.

5

56. The method of claim 53 where the substrate is

10 sufficiently high to saturate approximately all available substrate binding sites on any  $\beta$ -1,4 glucan synthetic enzyme contained within the cell-free extract to cause thereby the formation of extended sub-fibrils of the selected  $\beta$ -1,4 glucan polymer which sub-fibrils are of an approximately uniform width.

**FIG. 1A**

CGTTCCCGC ACATCAGCAT CGTCCATAAG TGAGCGTCCG GTGACAGGGT GTGCCCAGTG  
60

AATGGAGTCC TGTTC CGAAG CACGCCCTCAG CCGCGGGACG TCCGCGTCCC GCGATCTGA  
120

GCTTTTCTT TCAGGGGATG CGCGACACCC GTGATGAAA GAAGTTCTTT CCCGTGGCGT  
180

CCACGCGATC AGTTCGTTCT AATGTTTCCA GGATGACCAG CATGACCAAG ACAGACACGA  
240

ATTCCCTCTCA GGCTTCTCGT CCCGGCAGCC CCGTCGCCCTC GCCTGATGGG TCGCCCCACAA  
300

TGCCCCAAGT GTTCATGACG CTGGGTGGTC GTGCGACGGA ACTCCTCAGC CCCCCTCCTT  
360

CGCTGCGGGA GCGGCTGTTG CGTCGTCGTG AAAACGAAGA AGAATCCTAA GGCCCTATAT  
420

TCAGGCGACC GCCTGTCCCT GTGTCCTGCC TGGTGAAATG GCGGGGGCAG GGCAGACAC  
480

1 / 24

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**FIG. 1B**

GCTGCACTGG CTCGGCGATC TATCCGTCT TTGGCCTGGG TGATGTTTCG CAGCCCCAGG  
540

CGGGCCCGCT TTGCCCCGAT GTGACCGCAG ATGGTCGGG TCAGGTTTTC TTGACGTAA  
600

TTTTTCTGTT TTACAGCATT TCGGACGAGT TGTTT ATG CCA GAG GTT TGG 650  
Met Pro Gly Val Arg  
1

TCG TCA ACG CAG TCA GAG TCA GGA ATG TCA CAG TGG ATG GGC AAA 695  
Ser Ser Thr Gln Ser Glu Ser Gly Met Ser Gln Trp Met Glu Lys  
10 15 20

ATT CTT TCC ATT CGC GGT GCT GGT GGG CTG ACT ATT GGT GTT TTT GGC 740  
Ile Leu Ser Ile Arg Gly Ala Gly Leu Thr Thr Ile Gly Val Phe Gly  
25 30 35

CTG TGT GCG CTG ATT GCG GCT ACG TCC GTG ACC CTG CCG CCA GAA 785  
Leu Cys Ala Leu Ile Ala Ala Thr Ser Val Thr Leu Pro Pro Glu  
40 45 50

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**FIG. 1C**

CAG CAG TTG ATT GTG GCA TTT GTA TGT GTC GTG ATC TTT TTT ATT	830
Gln Gln Leu Ile Val Val Val Cys Val Val Ile Phe Phe Ile	65
	55
GTC GGT CAT AAG CCC AGC CGT CGG TCC CAG ATT TTC CTT GAA GTG	875
Val Gly His Lys Pro Ser Arg Arg Ser Gln Ile Phe Leu Glu Val	80
	70
CTG TCA GGG CTG GTT TCG CTG CGC TAT CTG ACA TGG CGC CTG ACG	920
Leu Ser Gly Leu Val Val Ser Leu Arg Arg Tyr Leu Thr Arg Leu Thr	95
	85
GAA ACG CTT TCA TTC GAT ACA TGG TTG CAG GGT CTG CTT GGG ACA	965
Glu Thr Leu Ser Phe Asp Thr Thr Trp Leu Gln Gly Leu Gly Thr	110
	100
ATG CTT CTG GTG GCG GAA CTT TAC GCC CTG ATG ATG ATG CTC	1010
Met Leu Leu Val Ala Glu Leu Leu Ala Leu Met Met Phe Leu	125
	115

3/24

SUBSTITUTE SHEET

4/24

**FIG. 1D**

AGC TAT TTC CAG ACG ATC GCG CCA TTG CAT CGT GCG CCT CTT CCG	1055
Ser Tyr Phe Gln Thr 130	Pro 140
CTG CCG CCG AAC CCT GAC GAA TGG CCC ACG GTC GAT ATC TTT GTC	1100
Leu Pro Pro Asn Pro 145	Val 155
CCG ACC TAC AAC GAA GAA CTG AGC ATT GTC CGC CTG ACG Thr Val	1145
Pro Thr Tyr Asn Glu Glu 160	Leu 170
GGA TCA CTG GCG ATT GAC GAC TGG CCA CCG GAA AAG GTG CCG GTT CAT	1190
Gly Ser Leu Gly 175	His 185
ATC CTT GAT GAC GGT CGT CGT Arg Arg 190	1235
Ile Leu Asp Asp Gly 190	Ala 200

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21 / 24

**FIG. 13C**

SUBSTITUTE SHEET

CTCGACGTACCGCGGTGGACCGTGTTCCGGCAGGATCAGTTGAACTTCTACTTTGACGCG	4380
L D V P P W T V F G Q D Q L N F Y F D A	507
ATGCCCTTGGCGCGGAATCTGTCAAAGTGGCGGCAACAATGCGTTCCATCTTGGG	4440
M P L A R G I C Q S G A A N N A F H L G	527
CTTGACCCGGATTCCACCATTGATTTTCCCGTGCCCATCACATCGCCAGATGCCCAAC	4500
L D P D S T I D F S R A H H I A Q M P N	547
CTTGCCCTATATGGCGACGGTCGGTTTCCCTTTCACCACCTATGCCGATCTGTGCAGACG	4560
L A Y M A T V G F P F T T Y A D L S Q T	567
GCGGTTGTTCTGCCTGAACACCCGAATGCCGCGACTGTTGGCGCCTATCTTGACCTGATG	4620
A V V L P E H P N A A T V G A Y L D L M	587
GGGTTCATGGCGCGGACGTGGTATCCGGTTGCGGGCGGTGGACATTTGIGTCGGCCGAT	4680
G F M G A A T W Y P V A G V D I V S A D	607
CATGTCAGTGACGTTGGGACCGTAACCTGCTGGTGATTTCGACGCTGGCGACAAGTGGC	4740
H V S D V A D R N L L V I S T L A T S G	627
GAGATCGCGCGCTGTACGTTTCATCCTACGAAGTGGCGGATGGTCAATTTCGCGCAG	4800
E I A P L L S R S S Y E V A D G H L R T	647
GTGTCGCATGCGTCCGCGTGGATAACGCGATCAAGCGGTGCGACGATCCGCTGACGGCT	4860
V S H A S A L D N A I K A V D D P L T A	667

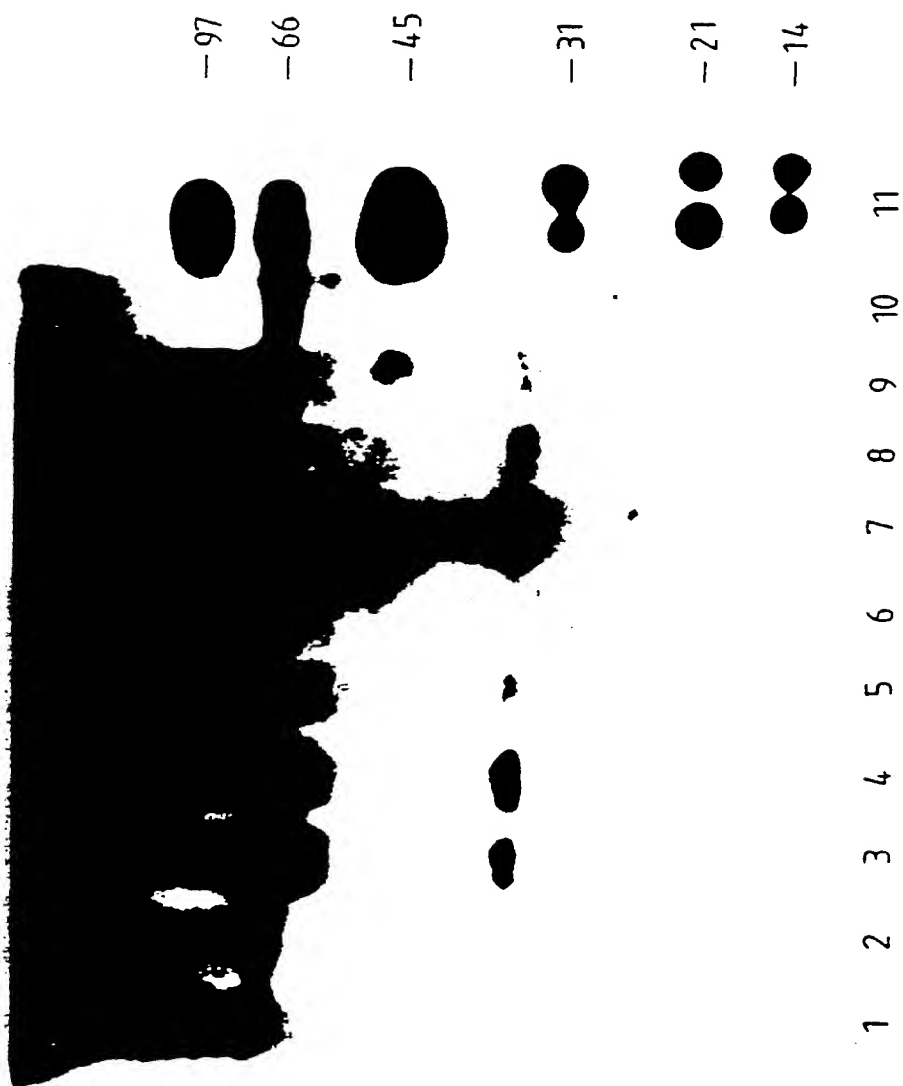
22 / 24

**FIG. 13D**

TTCCGCGACCGTGACAGCAAGCCGCGAGGATGTCGATACCCCCCTGACGGGTGTCGGC	4920
F R D R D S K P Q D V D T P L T G G V G	687
GCGATGATCGAGCGGAATCCCCCTGACGGCGCGCACCGTTCTGGCGCTGCTGTCG	4980
A M I E A E S P L T A G R T V L A L L S	707
TCTGACGGTGC GGGCTGAACAACCTCCCTTCAGATGTTGGGGAGCGCAAGAAGCAGGCG	5040
S D G A G L N N L L Q M L G E R K Q A	727
AATATCCAGGGGACCTGGTTGTTGCCCATGCGAGGACCTGTCTTCGTACCGCACTTCG	5100
N I Q G D L V V A H G E D L S S Y R T S	747
CCGGTCTATACGATCGGCACCCCTGCCGCTCTGGCTGTGGCCGACTGGTATATGCACAAC	5160
P V Y T I G T L P L W L W P D W Y M H N	767
AGACCGGTTGCTGCTGCTTGTTCGGCCCTGTGGGATGTATTCTGATCGTCAGTGTCTT	5220
R P V R V L L V G L L G C I L I V S V L	787
GCACGGGCTCTGGCGCGGCAATGCGACCCCGCGTTTCAAGCAGCTTGAGGATGAGAGGCGC	5280
A R A L A R H A T R R F K Q L E D E R R	807
AAGTCGTGACCCATAAACGATATGCTTCGTCCCTGTCCCGCGGTCTTCTCGCAACGACCT	5340
K S STOP	809
GCCTCGCAGGTCTGTGCTGCAG	5363

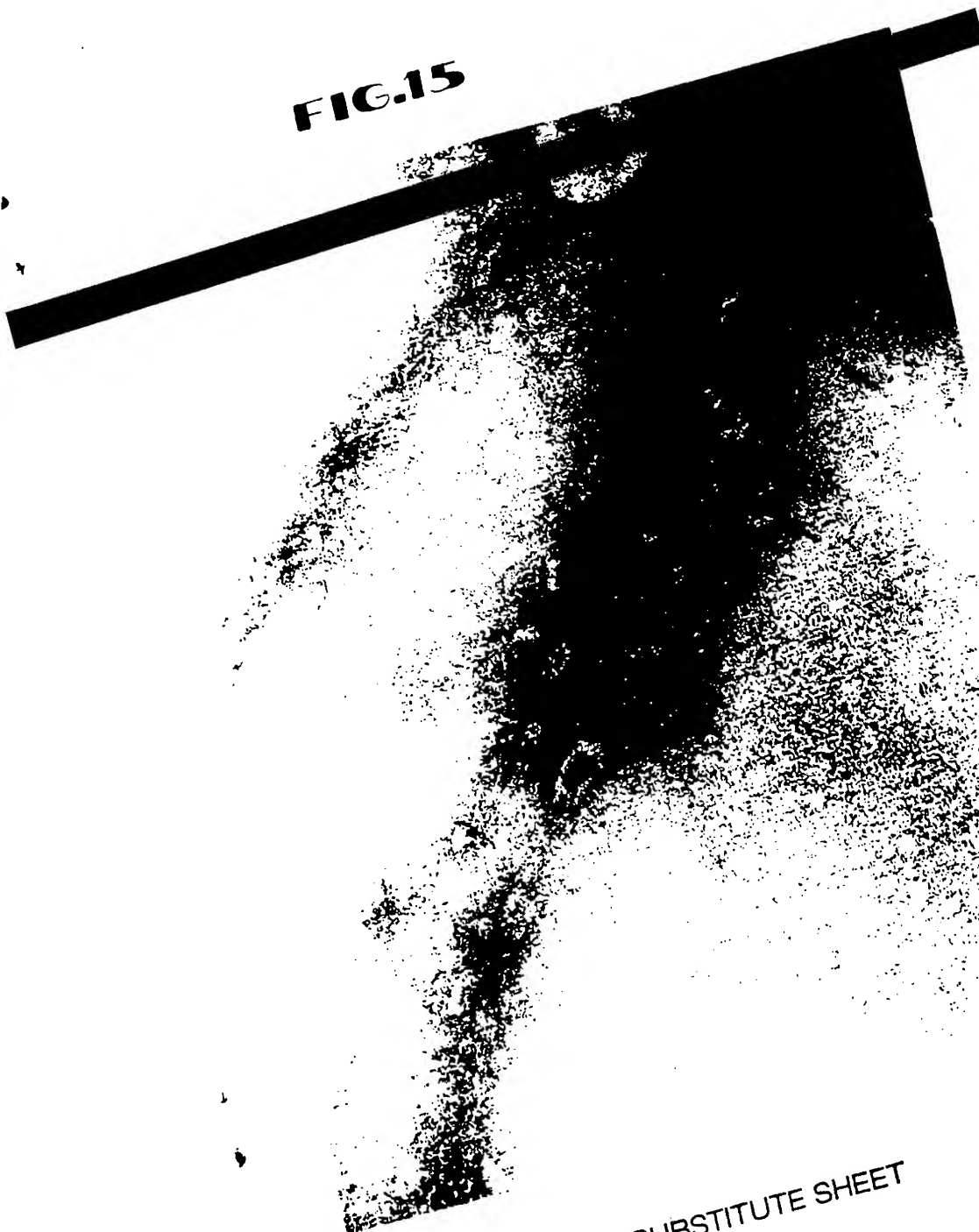
23 / 24

**FIG.14**



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**FIG.15**

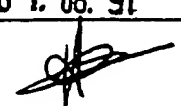


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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 91/01726

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5      C12N15/54 ;    A01H5/00 ;    C12P19/04 ;    C12Q1/68		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ;      C12R	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	LIN, F.C. & BROWN, R.M.: "Cellulose and wood - chemistry and technology" 1989, Schuerch, C., Ed., Wiley, New-York see pages 473 - 492	21-22, 24, 28, 30-31, 33-38
Y	(cited in the application)	1-20, 39, 42, 44
Y	US,A,4788135 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD, JR UNIVERSITY) 29 November 1988 see the whole document	1-20, 39, 42, 44
<div style="display: flex; justify-content: space-between;"> <div> <p><sup>10</sup> Special categories of cited documents : <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
21 JUNE 1991	01.08.91	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	ANDRES S.M. 	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	SCIENCE. vol. 230, 15 November 1985, LANCASTER, PA US pages 822 - 825; LIN, F.C. et al.: "Synthesis of fibrils in vitro by a solubilized cellulose synthase from Acetobacter xylinum" see the whole document	21, 31, 34-35, 37-38
X		49-51, 53-56
	(cited in the application)	32-33
Y	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 264, no. 20, 15 July 1989, BALTIMORE US DRAKE, R.R. et al.: "Synthesis and properties of 5-azido-UDP-glucose" pages 11928 - 11933 see the whole document (cited in the application)	28, 32-33
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 84, October 1987, WASHINGTON US pages 6985 - 6989; BUREAU, T.E. et al.: "In vitro synthesis of cellulose II from a cytoplasmic membrane fraction of Acetobacter xylinum" see the whole document (cited in the application)	21, 28, 31, 34-36, 49-51, 55
P,X	PLANT MOLECULAR BIOLOGY. vol. 15, 1990, DORDRECHT NL pages 673 - 683; SAXENA, I.M. et al.: "Cloning and sequencing of the cellulose synthase catalytic subunit gene of Acetobacter xylinum" see the whole document	1-3, 6-7, 9-11, 14, 16
		20-23, 28, 30-35, 37-39
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 265, no. 9, 25 March 1990, BALTIMORE US pages 4782 - 4784; LIN, F.C. et al.: "Identification of the uridine 5'-diphosphoglucose (UDP-Glc) binding subunit of cellulose synthase in Acetobacter xylinum using the photoaffinity probe 5-azido-UDP-Glc" see the whole document (cited in the application)	21, 28, 30-38, 49

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	<p>WO,A,9012098 (CETUS CORPORATION) 18 October 1990 see page 15, line 6 - page 19, line 13</p> <p>see page 37, line 15 - page 38</p> <p>see page 43, line 11 - page 46, line 30</p> <p>see page 51, line 25 - page 54, line 25 see pages 60 - 66, line 26 see page 78, line 13 - page 81, line 13; figure 1</p> <p>---</p>	<p>1, 6-7, 9, 20-21, 25, 28 30-31, 34-35, 37-40, 42-44 49-51, 53, 55</p>

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.

SA 46083

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
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21/06/91

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4788135	20-11-88		
WO-A-9012098	18-10-90	AU-A- 5437390	05-11-90

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82